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TISSUE CULTURE STUDIES WITH INFECTIOUS HEPATITIS SPECIMENS[1]

A. E. FRANKLIN[2] AND J. C. SINCLAIR[3]

Abstract

Twenty-nine human adult and eight monkey tissues were studied in culture. The addition of prednisolone to growth media restricted the growth to epithelial-like cells and also provided a slight growth stimulus. Thirty-four specimens of acute hepatitis origin failed to provide indications of virus. Detroit-6 cells were cultured in synthetic media supplemented with sheep serum. Sixteen experiments were inoculated with 82 specimens of acute hepatitis origin. There were no conclusive results with this line of cells.

Isolation of the etiological agent of infectious hepatitis remains a difficult problem. In 1956 we indicated that the use of human embryonic and monkey tissues did not provide a suitable method for detection of the virus (2). Pollard and Diserens (9) have described a cytological response in embryonated eggs to infectious hepatitis specimens, but Buchner *et al.* (1) have reported that the technique gave rise to erratic and inconsistent results. Rightsel *et al.* (10) obtained a cytopathogenic effect with infectious hepatitis specimens in Stulberg's Detroit-6 cells (12) but there has been no published confirmation. Recent serological tests by Hoyt and Morrison (7) and Rubin *et al.* (11) have been described using agglutination of *Macaca rhesus* erythrocytes. This paper describes the findings obtained with cultures of various human tissues, normal and neoplastic, monkey liver and spleen, and Detroit-6 cells, inoculated with specimens from patients with acute infectious hepatitis.

Methods and Procedures

Original cultures of human and monkey tissues were prepared and maintained in plasma-clotted roller tube cultures as described previously (2). Morgan's synthetic mixture No. 199 (8) was used in growth media in early experiments. We prepared a modified version of this mixture in which minor

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modifications of some biochemical constituents were made, and Earle's balanced salt solution was replaced with Tyrode's balanced salt solution. Mixtures 703, 858, and 1066, prepared by Healy *et al.* (5, 6), were used as a guide for our synthetic preparations. Most of the media used in our experiments were based on mixture No. 1066 with Tyrode's balanced salt solution. All growth media were supplemented with inactivated 10% horse or human sera. Maintenance media consisted of synthetic mixtures supplemented with 2% horse serum. Additional modifications in the media consisted of variations in the ratio of sodium chloride to potassium chloride in the balanced salt solution. All media were sterilized by Seitz filtration.

Cortisone was reported by Grossfield and Ragan (4, 3) to suppress the growth of fibroblasts in cultures of stomach and intestine without affecting epithelial growth. We have used prednisolone at a final concentration of 1 mg% in growth media for some liver and spleen cultures since 10 mg% was shown to cause widespread granularity in liver cells.

Sera and stool specimens obtained within 7 days of the onset of symptoms of infectious hepatitis (IH) were deep frozen as soon as possible. Serum specimens were used either undiluted (0.2 ml) or as a 1:5 dilution in isotonic saline. Stool specimens were used as 10% suspension and 0.1 ml inoculated. Controls consisted of normal and convalescent sera, obstructive jaundice sera, and stools obtained from persons with no clinical history of jaundice.

Experiments with Monkey Tissues

Preparation of Cultures

Eleven monkey tissues were obtained through the courtesy of the Connaught Medical Research Laboratories. The growth media consisted of synthetic mixtures supplemented with 10% horse serum. Four liver and three spleen cultures were subsequently inoculated with eight acute IH sera, one acute IH faecal extract, and three passaged specimens. The maintenance media consisted of the same synthetic mixtures supplemented with 2% horse serum.

Results

(1) Growth of monkey splenic tissue was rapid with confluent growth resulting in 5 to 7 days whereas liver outgrowths were slightly slower and not as extensive.

(2) When monkey liver was supplied with modifications of a synthetic mixture in which the ratio of sodium chloride to potassium chloride was varied, it was observed that excellent outgrowths could be obtained when the ratio was 6.8 g of NaCl to 0.4 g of KCl (standard), 5.1 g of NaCl to 2.1 g of KCl, and 3.4 g of NaCl to 3.8 g of KCl. The growth was much inferior when the ratio was 1.7 g of NaCl to 5.5 g of KCl and could not be obtained when sodium chloride was omitted from the medium and the potassium chloride level increased to 7.2 g per liter.

(3) There were no detectable differences between tissue cultures inoculated with acute IH sera and the corresponding convalescent sera.

Experiments with Human Tissues

Preparation of Cultures

Normal and abnormal human adult tissues were obtained at the time of operation or post mortem through the courtesy of the staff of the Toronto General Hospital, and consisted of the following specimens: breast, penis, meningioma, lung, liver, and spleen. Growth media consisted of synthetic mixtures supplemented with either horse or human serum. Prednisolone was added to the growth media in some experiments. The maintenance media consisted of synthetic mixtures supplemented with horse serum. Four cultures of neoplastic origin and one, presumably normal, were subsequently inoculated with seven acute IH sera; six liver cultures were inoculated with two acute IH sera, two acute-stage faecal extracts, and two tissue-culture passed specimens; and nine spleen cultures were inoculated with six acute IH sera and three tissue-culture passed specimens.

Results

(1) Twenty-nine (83%) of 35 human adult tissues provided outgrowths. The tissues from different organs showed considerable variation in their growth responses. Liver explants were the most difficult to grow. Tissues of neoplastic origin grew more rapidly and more abundantly than the corresponding normal tissues. Autopsy specimens had a high incidence of contamination, but one spleen specimen obtained 18 hours after death provided luxuriant outgrowths and was bacteriologically sterile.

(2) The addition of prednisolone to the growth medium tended to restrict the growth to epithelioid cells and to repress the growth of fibroblast-like and amoeboid cells. It also inhibited the formation of multinucleated syncytial cells in cultures of spleen. Prednisolone also tended to stimulate tissue growth, particularly in the case of human adult liver.

(3) Twenty experiments with cultures of various human tissues inoculated with 22 acute IH specimens failed to reveal any specific effects on either the first or subsequent passages.

Experiments with Detroit-6 Cells

Preparation of Cultures

Detroit-6 cells were obtained through the courtesy of Drs. Rightsel and McLean, of the Parke Davis Research Laboratories, Detroit, Michigan. Cell sheets were broken up with 1:5000 Versene in phosphate-buffered saline and 15,000 to 25,000 cells were added to cultures. The growth medium consisted of a synthetic mixture supplemented with 20% sheep serum. The maintenance medium consisted of (*a*) the same synthetic mixture with 5% horse serum or 10% sheep serum; (*b*) Hank's balanced salt solution with 15% sheep serum; (*c*) Tyrode's balanced salt solution with 15% sheep serum; and (*d*) Ringer-Lockes' balanced salt solution with 15% sheep serum or 15% horse serum. The inocula consisted of 10 acute IH sera and two acute-stage faecal extracts. These were compared with control specimens of eight convalescent sera, one obstructive jaundice serum, four "normal" sera, one

"normal" faecal extract, and uninoculated cultures. In other experiments, five acute IH sera were mixed with the corresponding convalescent specimens to neutralize a possible first passage cytopathogenic effect.

Results

(1) Almost 900 cultures were inoculated with a total of 82 specimens. Significant differences were not observed between the controls and the acute IH specimens, although a fairly high incidence of degeneration and cytopathogenic effects occurred at times in both groups.

The two acute-stage faecal extracts often produced a cytopathogenic effect in initial cultures but this could not be obtained in repeated second and third passages. Two acute IH sera were passaged four and five times without evidence of increasing degenerative effects. Therefore it was concluded that inoculation of Detroit-6 cells with acute-stage IH specimens did not yield conclusive results due to the erratic and inconsistent response of the cells.

Discussion

During the last 3 years, we have continued studies on the isolation of the etiological agent of infectious hepatitis using monkey and human tissues. Initially, human adult liver was difficult to grow. New growth media were investigated, and as the studies progressed, it became possible to grow almost every specimen of human liver. Monkey liver was reinvestigated with these media as well as spleen and numerous other human tissues, normal and neoplastic. None of these explants when inoculated with blood serum or faecal extracts from patients in the acute stage of infectious hepatitis gave evidence of a viral effect.

During this time, Rightsel and his colleagues obtained a cytopathogenic effect with infectious hepatitis specimens in Detroit-6 cells. We studied this line of cells for 9 months and found the results obtained were erratic and not always reproducible. The cells appeared to be very sensitive to animal sera, and frequently there would be widespread degeneration with control sera. These findings nullified the results obtained with infectious hepatitis specimens.

Epidemiological evidence and studies with human volunteers indicate a viral etiology despite the lack of laboratory proof. It is possible that the laboratory conditions of tissue culture so far devised are not suitable for a viral effect, or that the agent requires a potentiating factor or is in an inactive or prophase form. It is indeed a puzzling fact that the agent of infectious hepatitis does not give rise to degenerative changes in tissue cultures of human adult liver, when it is known that extensive changes may occur in liver cells during the course of the disease.

Conclusions

(1) Specimens of human adult and monkey tissues prepared in plasma-clotted tissue cultures have been grown in synthetic mixtures supplemented

with horse or human sera. The addition of prednisolone to the growth medium restricted the growth to epithelioid cells and also provided a slight growth stimulus.

(2) Monkey liver cultures were grown in media in which the level of sodium chloride to potassium chloride was varied over a wide range. Tissue growth was more inhibited as the level of the latter increased. There were no detectable differences under any of these conditions in cultures inoculated with acute-stage infectious hepatitis sera and the corresponding convalescent sera during a 2-month observation period.

(3) Seven experiments in which cultures of monkey liver and spleen tissues were inoculated with 12 specimens from patients with acute infectious hepatitis failed to indicate the presence of virus.

(4) Twenty experiments in which cultures of adult human tissues, normal, including liver, and neoplastic, were inoculated with 22 specimens from cases of acute infectious hepatitis did not yield a viral effect.

(5) Detroit-6 cells were cultured in synthetic mixtures supplemented with sheep serum. Sixteen experiments, involving almost 900 cultures, were inoculated with a total of 82 specimens from patients with acute infectious hepatitis. Widespread degeneration occurred with control sera and inconsistent findings frequently resulted from acute infectious hepatitis specimens both in primary and in passaged cultures. It was concluded that a specific viral effect was not obtained under our experimental conditions.

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UTILIZATION OF CARBOHYDRATES BY THE MOSQUITO FLAGELLATE, CRITHIDIA FASCICULATA[1]

WILLIAM B. COSGROVE

Abstract

The trypanosomid flagellate, *Crithidia fasciculata*, has been shown to have, in the absence of exogenous substrate, a long-sustained oxygen consumption (R.Q. 0.85) accompanied by the production of ammonia. Ribose, xylose, fructose, glucose, galactose, mannose, maltose, cellobiose, sucrose, raffinose, glycerol, and dihydroxyacetone abolish this oxygen consumption while increasing the rate of respiration up to fivefold. Glucose is incompletely oxidized (R.Q. 1.06) with the consumption of 2.6 moles of oxygen and production of 0.6 moles succinate and 0.4 moles ethanol and traces of pyruvate per mole of glucose consumed. Ethanol and a variety of other alcohols can be metabolized when presented as the only exogenous substrate. These results are discussed with respect to the metabolic activities of other members of the family Trypanosomatidae and to the hypothesis that the end products accumulate as a result of limiting amounts, rather than complete absence, of certain enzymes.

Introduction

The carbohydrate metabolism of numerous species of the genus *Trypanosoma* has been investigated (e.g. Ryley (7)) but little attention has been devoted to the less specialized genera which inhabit invertebrates and plants. This study constitutes part of a detailed investigation of the metabolism of *Crithidia fasciculata*,[2] a parasite of mosquitoes, which has been the subject of extensive nutritional studies (Cowperthwaite *et al.* (2); Kidder and Dutta (5)).

Materials and Methods

The strain of *C. fasciculata* used is the same as that of Cowperthwaite *et al.* The organisms were grown axenically on their crude medium, with sorbitol, at 25° C and the cultures harvested during the logarithmic phase. The organisms were washed by centrifugation three times with amphibian Ringer buffered at pH 7.6 with triethanolamine, followed by two washings with Ringer-phosphate. The packed organisms were then suspended in Ringer-phosphate for use.

Carbohydrates, obtained from Nutritional Biochemicals and from California Corporation for Biochemical Research, were made up as 0.2 *M* stock solutions in a mixture of two parts of Ringer-triethanolamine and one part Ringer-phosphate, and stored frozen. Stock solutions of the alcohols (Eastman grade) were made up just before use.

[1]Manuscript received June 16, 1959.
Contribution from the Department of Zoology, University of Georgia, Athens, Georgia, U.S.A. Part of this investigation was completed while the author was a member of the Department of Zoology, State University of Iowa.

[2]The differences between the present data for *C. fasciculata* and those of Ryley (6) for *Strigomonas oncopelti* seem sufficiently great to controvert Seaman's (10) suggestion that the two flagellates represent different strains rather than different species.

Oxygen consumption was measured by the direct Warburg technique and the R.Q. determined by the first method of Dickens and Simer. The estimation of glucose and end products was made on the neutralized contents of the Dickens–Simer cups.

The analytical methods listed by Ryley (6) were used with the following exceptions: glucose was determined, in the presence of other reducing sugars, by the colorimetric glucose oxidase technique (Saifer and Gerstenfeld (8)); ethanol, following distillation from acid and redistillation from alkali, by the method of Williams and Reese (12). Hexoside hydrolase activity was determined according to Hestrin *et al.* (3).

All experiments were carried out at 25° C.

Results

This flagellate has an appreciable respiration (Q_{O_2} (N) = −135) which continues undiminished for 3 to 4 hours after the removal of exogenous substrates. The substrate for this respiration was investigated by analyzing masses of flagellates and their suspending medium before and after 10 hours of respiration in the absence of exogenous substrate, and by the determination of R.Q. during the first and tenth hours. The results are presented in Table I, and indicate that a nitrogenous material is the substrate. This material cannot be triethanolamine because the same results are obtained when it is omitted from the medium.

TABLE I

Metabolism of endogenous substrate

A. Respiratory activity

	1st hour	10th hour
R.Q.	0.85	0.85
μl O_2 per hour per 10^8 organisms	467	404

B. Chemical analysis

(All values are expressed as milligrams per 10^8 organisms)

	Initial	Final
Lipid	2.99	3.06
Reserve carbohydrate (as glucose)	0.121	0.119
Total nitrogen	3.52	3.51
Ammonia nitrogen	0.05	0.38

Table II presents the results of studies on the stimulation of oxygen consumption by various carbohydrates and related compounds. As in all experiments of this sort, there is a possibility that failure of a particular carbohydrate to stimulate is due to impermeability of the membrane rather than to the absence of the enzymes required for its utilization. This possibility

TABLE II

Stimulation of oxygen consumption by carbohydrates

Substrate, 0.02 *M*	Q_{O_2} (N) in % of rate in absence of exogenous substrate*
Arabinose	100 (2.1)
Ribose	320 (5.4)
Xylose	123 (1.8)
Fructose	486 (11.0)
Galactose	377 (6.2)
Glucose	525 (19.0)
Mannose	530 (11.2)
Maltose	311 (7.5)
Cellobiose	133 (1.8)
Lactose	100 (3.1)
Melibiose	100 (2.9)
Sucrose	381 (7.4)
Trehalose	99 (2.5)
Turanose	104 (4.1)
Raffinose	224 (5.3)
Glycerol	298 (9.6)
Dihydroxyacetone	220 (10.1)

*Mean of five determinations (standard error).

seems slight in these experiments because of the similarity of molecular size and chemical properties of certain stimulating and non-stimulating sugars. The use of cell-free extracts and acetone powders to circumvent the problem of permeability was impossible because these preparations show no stimulation of oxygen consumption even with glucose as a substrate. The cell-free extracts do show strong hexoside hydrolase activity with sucrose and maltose as substrates, weak activity with cellobiose, and no activity with lactose or melibiose.

The possibility that the smaller stimulation given by, for example, cellobiose might be due to readily metabolizable impurities seems to be ruled out by the combined findings that the amount of stimulation was the same with both brands of a carbohydrate and that reducing the concentration of the carbohydrate did not affect the amount of stimulation at concentrations above 0.002 *M*.

Ten determinations of the R.Q. and of the molar ratio of quantity of oxygen consumed to that of glucose utilized gave mean values of 1.06 ± 0.012 and of 2.60 ± 0.05 respectively. This R.Q. is significantly different from 1.00 at the 1% level. The molar ratio is greatly different from the value of 6 representing complete oxidation of glucose, indicating that appreciable quantities of incompletely oxidized end products are formed. Qualitative chromatographic analysis of incubation mixtures indicated that the end products consisted of pyruvic and succinic acids and ethanol. The results of quantitative investigations are shown in Table III; in both experiments, essentially all of the carbon of the glucose has been accounted for.

The chance observation that when nearly all of the glucose had been metabolized, the ethanol yield was less than that predicted from the data of

TABLE III

End products of glucose oxidation

	Experiment I			Experiment II		
	A	B	C	A	B	C
Glucose	−12.1	—	—	− 6.56	—	—
O_2	−31.2	2.58	—	−16.9	2.58	—
CO_2	+33.0	2.73	2.73	+17.3	2.64	2.64
Pyruvate	+0.48	0.04	0.12	+0.26	0.04	0.12
Succinate	+7.25	0.60	2.40	+4.06	0.62	2.48
Ethanol	+4.71	0.39	0.78	+2.43	0.37	0.74
Total			6.03			5.98

NOTE: Conditions: Each Dickens–Simer flask contained 1.0 ml washed organisms (0.5–0.75 mg nitrogen), 2.0 ml Ringers, pH 7.6, containing 60 μmoles glucose; side arm contained 0.5 ml 3 *N* HCl; trough, 0.5 ml 3 *N* NaOH (carbonate-free); gas phase air.
A, micromoles consumed (−) or produced (+) per flask;
B, micromoles per micromole glucose consumed;
C, atoms carbon recovered per molecule glucose consumed.

TABLE IV

Stimulation of oxygen consumption by alcohols

Substrate (molarity)	Q_{O_2} (N) in % of rate in absence of exogenous substrate*
Methyl (0.02)	101 (2.3)
Ethyl (0.02)	301 (5.2)
n-Propyl (0.02)	197 (8.4)
Isopropyl (0.02)	97 (1.8)
n-Butyl (0.01)	202 (10.3)
Isobutyl (0.01)	201 (8.6)
sec-Butyl (0.01)	106 (1.5)
tert-Butyl (0.01)	95 (3.2)
n-Amyl (0.005)	240 (16.0)
Isoamyl (0.005)	110 (0.7)
tert-Amyl (0.005)	103 (1.5)
Diethyl carbinol (0.005)	99 (2.1)
sec-Butyl carbinol (0.005)	140 (4.4)
Methyl isopropyl carbinol (0.005)	105 (4.1)
n-Hexyl (0.002)	130 (5.8)

*Mean of five determinations (standard error).

Table III prompted an investigation of ethanol and other alcohols as substrates. The results shown in Table IV indicate that a number of alcohols stimulate oxygen consumption.

Discussion

The occurrence of a long-sustained oxygen consumption, in the absence of exogenous substrate, in this flagellate represents an unusual, though not unique, condition among trypanosomids. Ryley (6) reported that *Strigomonas oncopelti* is able to sustain a constant rate of oxygen consumption for several hours in the absence of exogenous substrate, and that the endogenous substrate cannot be glycogen. The present results with *C. fasciculata* indicate that in this species the endogenous substrate is a nitrogenous coumpound. By

using the production of ammonia as an index of the intensity of this respiration, it could be shown that all of the utilizable sugars abolish respiration based on endogenous substrate.

The reserve carbohydrate material of Table I does not represent glycogen; this material is readily isolated by the standard Somogyi procedure for glycogen, but upon acid hydrolysis yields reducing substances which are not fermented by yeast and are inactive as substrates for glucose oxidase. The nature of this reserve carbohydrate is now under investigation.

The ability to use only certain oligosaccharides is understandable from the nature of the hydrolases present, for example galactosidases are lacking and neither lactose nor melibiose is utilized. The presence of an α-glucosidase is shown by utilization of maltose. Raffinose, a melibiose β-fructoside, is hydrolyzed to melibiose and fructose, as shown by chromatographic analysis, indicating that a β-fructosidase (= "invertase") is also present. This β-fructosidase should also be capable of hydroylzing sucrose, but the relative importance of it and of α-glucosidase in the utilization of sucrose cannot be assessed. The somewhat greater stimulation by sucrose than by maltose might be interpreted as the result of more rapid hydrolysis resulting from the presence of two hydrolases able to act on sucrose. Further comment on hydrolase activity seems unwarrented with data from crude extracts. The failure to utilize trehalose is surprising in view of the report of Wyatt and Kalf (13) that trehalose is the major carbohydrate in the blood of certain insects, including *Anopheles quadripennis.*

Among the monosaccharides, where hydrolase activity cannot be involved, the ready utilization of the hexoses indicates that they are interconvertible, presumably by mechanisms similar to those of vertębrate tissue. Schwartz (9) has presented evidence for the operation of the hexose monophosphate shunt in this flagellate. The inability to use arabinose must then be interpreted as a failure to phosphorylate this pentose; once phosphorylated, the monophosphate shunt should provide a pathway for conversion to ribose or xylose, both of which are readily oxidized. L-Arabinose, more common in nature, also is inactive.

The ability of *C. fasciculata* to use a wide variety of alcohols is in sharp contrast to the restriction to a few monosaccharides reported for some species of *Trypanosoma.* In this ability, *C. fasciculata* resembles *S. oncopelti* more than the trypanosomes. No attempt has been made to investigate the metabolic pathways of these alcohols; failure to use those isomers in which oxidation of the hydroxyl carbon cannot proceed through carboxyl formation without rupture of the carbon chain indicates an initial oxidation to the corresponding aliphatic acid.

In its inability to oxidize glucose completely, *C. fasciculata* conforms to the categorization of the trypanosomids as aerobic fermenters (von Brand (11)). It does not form as great a variety of end products as *S. oncopelti* (Ryley (6)) but like this species produces considerable amounts of ethanol. About 45% of the carbon metabolized appears as CO_2 and about 41% as succinic acid. Hunter's (4) results indicate that the accumulation of these end products

is not the result of qualitatively different metabolism. In all probability, it is rather the result of a quantitative deficiency of certain intermediary enzymes with the resulting accumulation of their substrates when the initial substrate is present in relatively high concentration. When the concentration of the initial substrate is too low to saturate the initial reaction system, intermediates are not formed rapidly enough to saturate the deficient enzyme systems and the previously formed intermediates which have accumulated are now metabolized; for example, ethanol begins to be utilized as glucose nears exhaustion. Succinate is not used because, in its ionized form at pH 7.6, it is unable to enter the cell. In a single experiment run at pH 3, some of the accumulated succinate was utilized as the glucose neared exhaustion. This explanation may also apply to apparent changes in ability to utilize acidic materials associated with changes in the dominant morphological form in cultures. For example, Chang (1) reported that, in cultures of *Trypanosoma cruzi*, the oak-leaf forms appearing first in the culture lower the pH by the formation of acidic end products which are consumed by the crithidia and trypanosome forms developing later.

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SEED-BORNE FUNGI IN RELATION TO COLONIZATION OF ROOTS[1]

E. A. PETERSON

Abstract

As part of a study of the microflora developing on plant roots, analyses were made of the fungal populations of non-sterilized barley, flax, and wheat seed and of their primary roots 2 days after seeding in natural soil and at short intervals thereafter over a period of 3 weeks. It was found that fungi associated with the seed played little part in the colonization of roots in the soil. Species of *Aspergillus*, *Penicillium*, and *Alternaria*, abundant on the seed, were rarely obtained from root samples. The young roots (2 days after seeding) were relatively free from fungi; however, those which were colonized at this time yielded species of *Pythium* and *Fusarium* predominantly. In subsequent samples the percentage incidence of *Pythium* declined markedly, the predominating types being species of *Fusarium*, *Phoma*, *Pullularia*, *Periconia*, and *Cylindrocarpon*, the latter genus assuming greater prominence on wheat than on either barley or flax.

Introduction

Research on rhizosphere microbiology has been directed largely towards studies on the influence of plants on the microorganisms of the soil. However, in 1951, after analysis of the seeds of certain agricultural crops, Wallace and Lochhead (7) postulated that rhizosphere bacteria constitute a group intermediate between the indigenous soil bacteria and the flora of the seed. Attention was drawn to the possible contribution of the seed microflora to the rhizosphere population. Rovira (6) has since demonstrated that seed coat bacteria are capable of establishing themselves on the root surface; however, as his plants were grown on sterilized beads or sand, the natural soil microflora was not involved. Recently, Rouatt (5) compared the microflora of wheat seed with that of young seedling roots. He reported that certain bacteria such as denitrifying, gelatin-liquifying, and cellulose-decomposing types, not found on the seed, were prominent in the rhizosphere. Although the rhizosphere microflora was likely derived from both seed and soil, it was considered that most of these organisms originated in the soil.

The role of seed-borne fungi in colonization of roots is perhaps best understood in terms of specific plant pathogens. Yet, the seeds of crop plants are known to carry a wide range of fungal species (1, 2, 3), most of which are apparently innocuous inhabitants of the seed (3). Little is known about their importance as root colonists in the soil. A comparative study of seed and root fungal populations was therefore undertaken to determine whether seeds are the primary source of the fungi which become associated with the roots of healthy plants in soil.

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Methods

Barley, flax, and selected varieties of wheat[2] were sown in beds of a fertile field soil in the greenhouse. To determine fungal types associated with the seed, a 10-g sample of untreated seed was macerated in 100 ml of sterile water in a Waring blendor for 3 minutes. Platings were made from suitable dilutions of the homogenate. Root samples for fungal analysis were taken 2 days after seeding and thereafter at short intervals over a period of 3 weeks. For each root sample, about 60 seedlings were removed from the soil. One 4-cm length of root, taken immediately below the crown, was cut from each seedling (the whole root, averaging 2 cm, was used for the 2-day sample). After removal of lateral rootlets, the root lengths were serially washed in 20 changes of sterile water (4). Only two 0.5-cm segments cut from opposite ends of each length of root were plated.

Peptone dextrose agar containing rose bengal and chlortetracycline (4) was used for primary isolation of fungi. Subcultures for identification were made on potato dextrose agar slants. All plates were incubated at 20° C and were retained for at least 3 weeks to permit isolation of any slowly developing types.

Results and Discussion

The predominant genera of fungi obtained from the seed and young roots of barley and flax are shown in Table I. It may be noted that the main types of fungi found associated with the seed samples were either entirely absent from the seedling roots or their proportions were relatively small. For example, species of *Aspergillus* predominating on the barley seed were not obtained from the barley seedling roots. Similarly, *Alternaria* spp. obtained in abundance from flax seed occurred in low proportions on the flax roots 2 days after seeding, but were not found in subsequent samples.

In general, the types of fungi attaining predominance on the young roots were absent from the seed samples. Although not indicated in the table, many of the root segments taken 2 days after seeding were free from fungi, 77 and 58%, respectively, for barley and flax. However, almost all segments from samples taken after 5 days were found to be colonized. Root segments which were colonized as early as 2 days after seeding yielded species of *Pythium* and *Fusarium* predominantly. In subsequent samples the percentage incidence of *Pythium* decreased markedly, the predominating types being species of *Fusarium*, *Phoma*, *Pullularia*, *Periconia*, and *Cylindrocarpon*. Conspicuous increases in the relative abundance of *Fusarium* and *Phoma* were noted as early as 9 days after seeding.

The fact that certain dark, yeast-like fungi classified as *Pullularia* spp. were obtained both from the seed and from the young roots of barley does not necessarily implicate the seed as their source. It may be noted that relatively high proportions of these fungi also occurred on the roots of flax, yet they were

[2]Data presented for wheat are based on independent experiments conducted after completion of the study with barley and flax.

TABLE I

Percentage incidence of predominant fungi associated with seed and young roots of barley and flax

Plant	Genus	Seed	Roots				
			2*	5	9	14	20
Barley	*Pythium*	—	22.0	—	3.1	1.6	4.9
	Phoma	—	2.4	31.3	26.7	20.6	29.2
	Aspergillus	40.0	—	—	—	—	—
	Penicillium	21.0	—	3.6	—	—	1.1
	Pullularia	8.0	9.8	8.4	2.5	4.8	3.2
	Periconia	—	—	1.2	0.6	7.9	11.9
	Alternaria	15.0	—	—	—	—	—
	Fusarium	—	22.0	24.1	54.7	52.9	40.0
	Cylindrocarpon	—	7.3	2.4	3.1	3.2	5.4
Total no. of isolates		100	41	83	161	189	185
Flax	*Pythium*	—	68.9	1.2	1.2	2.1	0.5
	Phoma	0.8	—	21.0	34.7	36.1	22.5
	Aspergillus	—	—	—	—	—	—
	Penicillium	4.8	—	1.2	1.2	—	0.5
	Pullularia	—	—	17.3	10.4	18.0	18.7
	Periconia	—	—	—	8.7	7.2	12.1
	Alternaria	84.7	2.8	—	—	—	—
	Fusarium	—	12.3	29.6	33.5	30.4	31.9
	Cylindrocarpon	—	0.9	—	5.8	0.5	2.7
Total no. of isolates		124	106	81	173	194	182

*Days after seeding.

TABLE II

Percentage incidence of predominant fungi associated with seed and young roots of wheat

Variety	Genus	Seed	Roots		
			2*	9	20
Saunders	*Pythium*	—	68.4	—	1.3
	Phoma	—	—	32.3	30.0
	Aspergillus	38.2	—	—	—
	Penicillium	30.3	—	—	—
	Pullularia	—	5.3	4.6	16.3
	Periconia	—	—	3.1	3.8
	Cladosporium	4.5	—	—	—
	Alternaria	9.0	—	—	—
	Fusarium	—	10.5	18.5	26.3
	Cylindrocarpon	—	—	13.8	13.8
Total no. of isolates		89	19	65	80
Selkirk	*Pythium*	—	51.7	—	—
	Phoma	—	—	15.9	24.4
	Aspergillus	1.0	—	—	—
	Penicillium	—	6.9	1.6	—
	Pullularia	2.0	3.4	1.6	1.3
	Periconia	—	—	—	5.1
	Cladosporium	58.0	3.4	—	—
	Alternaria	27.0	—	—	—
	Fusarium	4.0	3.4	33.3	23.1
	Cylindrocarpon	—	—	17.5	12.8
Total no. of isolates		100	29	63	78

*Days after seeding.

not obtained from flax seed. One culture assigned to the genus *Phoma* which was obtained from flax seed did not resemble any of the *Phoma* spp. isolated from the roots.

Results similar to those described above were obtained with four varieties of wheat grown in the same soil after completion of the barley and flax tests. Representative data for two varieties are shown in Table II. Although the proportions of fungal types obtained from seed differed with variety, the general pattern of root colonization was found to be essentially the same for all varieties studied. It is of interest to note that species of *Cylindrocarpon* assumed greater prominence on wheat than on either barley or flax.

The foregoing data, the results of two independent experiments, appear to justify the conclusion that soil is the primary source of the fungi which colonize the roots of healthy plants.

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THE INDUCTION BY ALLYL GLYCINE OF HERITABLE RESPIRATORY DEFICIENCY IN SACCHAROMYCES AND ITS REVERSAL BY SULPHUR AMINO ACIDS[1]

ALVIN SARACHEK AND GREGORY L. FOWLER

Abstract

Respiration-deficient mutants can be induced in populations of *Saccharomyces* growing in the presence of the cysteine antagonist, allyl glycine. Mutations occur, however, only among cells which had been cultivated anaerobically so as to be unadapted to aerobic respiration prior to exposure to the antagonist. The mutagenicity of allyl glycine is relieved by cysteine, and to a lesser extent by homocysteine, methionine, or alpha-amino-*n*-butyric acid. It is accentuated by homoserine. Evidence is presented indicating that the ability of allyl glycine to induce respiratory mutants is distinct from its general growth-inhibiting properties. It is concluded that the maintenance of the genetic determinant of respiration in *Saccharomyces* depends upon a particular metabolic function in which cysteine participates. In cells cultivated anaerobically that function is so limited that it can be rendered effectively inactive by growth in the presence of allyl glycine.

Introduction

To date, efforts to determine the physiological origin of cytoplasmically inherited respiratory deficiency in *Saccharomyces* have centered about the identification of particular chemical and physical agents capable of inducing the defect. Studies with the most effective inducers, acridine dyes (9), ultraviolet radiation (6), elevated incubation temperatures (12), and cation imbalance (8, 11), have established that the significant site of mutagenesis resides within some system peculiarly concerned with the adaptive formation of respiratory enzymes. However, since each of these agents causes a variety of metabolic disturbances within the cell, their identification has provided little insight into the nature of the particular biochemical lesion responsible for the mutation.

Recently, the suggestion was made that manganous ions can cause heritable respiratory deficiency in yeasts by instigating aberrant protein syntheses (7). While exploring this question further with the use of various amino acid analogues it was discovered that one analogue, allyl glycine, is itself capable of inducing the mutation. Its activity, however, is restricted to cells which are unadapted to aerobic respiration. The present report describes the conditions under which allyl glycine exerts its mutagenicity and presents evidence that the genetic maintenance of respiratory competence by yeast cells is specifically dependent upon the metabolism of the sulphur amino acids.

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Materials and Methods

An adenine-requiring tetraploid strain of *Saccharomyces* was employed as the test organism. Respiration-deficient mutants of spontaneous origin occur within this strain in a frequency of about 0.3%.

The general experimental procedures were as follows. Cells were cultivated upon a synthetic medium under either aerobic or anaerobic conditions so as to be adapted or unadapted to aerobic respiration. They were then transferred while in log phase to fresh growth media containing allyl glycine with or without amino acid adjuncts. Following a 22-hour period of either aerobic or anaerobic incubation at 28° C samples were removed and plated to determine increases in cell numbers and the frequencies of respiratory mutants appearing in the populations. The methods for the aerobic and anaerobic cultivation of cells and a quantitative evaluation of their effects upon the state of cellular respiratory activity have been described previously (6). Other details concerning the exposure of cells to the mutagen and the technique for scoring respiratory mutants have been recorded elsewhere (7).

The synthetic growth medium was of the following composition: glucose, 40 g; ammonium tartrate, 2 g; KH_2PO_4, 1.5 g; K_2HPO_4, 0.3 g; $MgSO_4.7H_2O$, 0.5 g; $CaCl_2$, 0.33 g; $FeSO_4.7H_2O$, 3 mg; $ZnSO_4.7H_2O$, 3 mg; $CuSO_4.5H_2O$, 0.5 mg; $MnSO_4.4H_2O$, 0.4 mg; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.15 mg; $Na_2B_4O_7.10H_2O$, 0.9 mg; inositol, 10 mg; thiamine hydrochloride, 1 mg; pantothenic acid, 0.6 mg; para-aminobenzoic acid, 0.6 mg; pyridoxine, 0.6 mg; nicotinic acid, 0.6 mg; biotin, 0.02 mg; adenine sulphate, 150 mg; water, 1000 ml. When used as a plating medium the glucose and adenine concentrations were reduced to 20 g and 40 mg per liter respectively and 1 g of casamino acids (Difco) and 20 g of agar were added per liter. Amino acids were procured from the Nutritional Biochemical Corporation; they were added to culture media prior to autoclaving at 10 pounds pressure for 10 minutes.

Results

Inhibition of Growth and Induction of Respiratory Variants by Allyl Glycine

Preliminary experiments established that allyl glycine has no selective effect upon the relative growth rates of respiration-deficient mutants and normal cells. Therefore, increases in mutant frequencies appearing in normal cell populations exposed to allyl glycine can be attributed directly to the mutagenicity of the analogue. The data summarized in Table I express the mutagenic and growth-inhibiting properties of allyl glycine in terms of the state of adaptation to aerobic respiration of cells prior to their exposure to the compound and the extent of aerobiosis existent during exposure. It can be seen that the conditions affecting inhibition of growth and mutation differ significantly in two ways. Firstly, growth inhibition is totally independent of the respiratory circumstances of the cells treated; the induction of respiration-deficient mutants, however, can occur *only* among cells which were unadapted to aerobic respiration before treatment. Furthermore though these unadapted cells mutate during either aerobic or anaerobic exposure to the

TABLE I

The effects of varying concentrations of allyl glycine upon the extent of growth and the frequency of respiratory mutants obtained in cultures inoculated with cells adapted or unadapted to aerobic respiration and incubated for 22 hours under either aerobic or anaerobic conditions

dl-Allyl glycine concentrations, molar	Respiration-adapted inoculum*				Respiration-unadapted inoculum†			
	Aerobic incubation		Anaerobic incubation		Aerobic incubation		Anaerobic incubation	
	Cell count/ml	% mutants	Cell count/ml	% mutants	Cell count/ml	% mutants	Cell count/ml	% mutants
2×10^{-2}	5.4×10^{4}	0.3	5.2×10^{4}	0.3	4.9×10^{4}	16.3	4.7×10^{4}	32.2
1×10^{-2}	6.7×10^{4}	0.4	6.3×10^{4}	0.4	6.6×10^{4}	22.5	6.4×10^{4}	41.3
5×10^{-3}	7.8×10^{4}	0.3	8.3×10^{4}	0.3	7.2×10^{4}	49.4	7.4×10^{4}	57.0
2×10^{-3}	9.6×10^{4}	0.2	9.8×10^{4}	0.3	9.2×10^{4}	11.8	9.0×10^{4}	43.0
1×10^{-3}	1.3×10^{5}	0.3	1.4×10^{5}	0.3	1.2×10^{5}	2.4	1.3×10^{5}	27.9
5×10^{-4}	1.8×10^{5}	0.4	1.9×10^{5}	0.4	1.6×10^{5}	0.8	1.8×10^{5}	4.6
None	2.8×10^{7}	0.3	4.5×10^{6}	0.4	2.2×10^{7}	0.3	4.1×10^{6}	0.3

*Cultures inoculated to an initial density of 4.5×10^{4} cells/ml.
†Cultures inoculated to an initial density of 4.1×10^{4} cells/ml.

analogue, their responsiveness is enhanced by the anaerobic exposure. Evidently the cells unadapted to aerobic respiration are deficient in one or more functions especially required for the genetic maintenance of the aerobic respiratory apparatus and the presence of allyl glycine suffices to make this deficiency an absolute one. Secondly, over the range of allyl glycine concentrations tested, the extent of growth inhibition is directly proportional to the analogue's concentration. In contrast, the induction of respiratory variants is optimal in the presence of 5×10^{-3} *M* allyl glycine and declines in the presence of higher as well as lower concentrations. These relationships would indicate that, during growth, the syntheses responsible for the stability of the genetic determinant of respiration are separable from those involved in other cellular activities and that the respiration-deficient state may arise from the imbalanced growth resulting from their selective disturbance. Comparable conclusions were reached in a previous study of the induction of respiratory deficiency in yeast by manganous ions (7).

Reversal of Allyl Glycine Activity by Sulphur Amino Acids

Dittmer *et al.* (1) have proposed, on the basis of structural considerations, that allyl glycine is a biological antagonist of cysteine. Accordingly, cysteine and those amino acids biosynthetically related to cysteine were tested over a range of concentrations for their abilities to relieve both the inhibition of growth and the mutation occurring among respiration-unadapted cells exposed aerobically to 5×10^{-3} *M* allyl glycine. The results summarized in Table II show that inhibition of growth can be reversed by cysteine, homocysteine, or methionine. In each instance optimal reversal occurs when the amino acid is in the same concentration as the analogue. None of the amino acids, however, is able to restore growth to the level of an allyl glycine free control culture. Such failure could be ascribed either to an incomplete relief of the toxicity of allyl glycine or to an additional toxicity contributed by the sulphur amino acids at the concentrations employed. To evaluate these alternatives

TABLE II

The abilities of varying concentrations of cysteine or amino acids biosynthetically related to cysteine to alleviate growth inhibition and the induction of respiratory variants by 5×10^{-3} *M dl*-allyl glycine. Cultures were inoculated with respiration-unadapted cells to a density of 4.2×10^{4} cells/ml and then incubated aerobically for 22 hours

Amino acid		Molar concentrations					
		2×10^{-2}	1×10^{-2}	5×10^{-3}	2×10^{-3}	1×10^{-3}	5×10^{-4}
dl-Alpha-amino-*n*-butyric acid	Cell count/ml	6.4×10^{4}	6.8×10^{4}	6.3×10^{4}	6.8×10^{4}	7.1×10^{4}	—
	% mutants	0.3	6.6	12.5	29.2	39.8	
dl-Cysteine	Cell count/ml	1.4×10^{5}	1.5×10^{5}	5.6×10^{5}	1.7×10^{5}	1.1×10^{5}	8.9×10^{4}
	% mutants	0.3	0.3	0.3	0.3	0.3	1.4
dl-Homocysteine	Cell count/ml	8.3×10^{4}	9.7×10^{4}	2.6×10^{5}	1.4×10^{5}	1.0×10^{5}	9.7×10^{4}
	% mutants	0.4	0.3	0.3	0.3	1.1	4.4
dl-Homoserine	Cell count/ml	6.9×10^{4}	6.6×10^{4}	7.4×10^{4}	7.2×10^{4}	6.5×10^{4}	6.8×1^{4}
	% mutants	93.6	70.2	54.9	42.2	44.3	41.1
dl-Methionine	Cell count/ml	2.0×10^{5}	3.8×10^{5}	6.8×10^{5}	3.3×10^{5}	2.4×10^{5}	9.6×10^{4}
	% mutants	0.3	0.3	0.3	0.9	1.8	5.7
dl-Serine	Cell count/ml	7.1×10^{4}	6.9×10^{4}	6.4×10^{4}	7.3×10^{4}	7.1×10^{4}	—
	% mutants	40.9	38.7	43.6	45.2	41.9	
dl-Threonine	Cell count/ml	6.9×10^{4}	6.9×10^{4}	6.8×10^{4}	7.1×10^{4}	6.7×10^{4}	—
	% mutants	43.0	48.2	47.7	49.1	43.6	

Controls

A. 5×10^{-3} *M dl*-allyl glycine and no amino acid supplement
Cell count/ml 6.5×10^{4}
% mutants 45.0

B. No allyl glycine and no amino acid supplement
Cell count/ml 2.6×10^{7}
% mutants 0.3

TABLE III

Comparisons of the extent of growth obtained after 22 hours in aerobic cultures containing 5×10^{-3} *M* cysteine, homocysteine, or methionine with or without 5×10^{-3} *M* allyl glycine. Cultures were inoculated initially with respiration-unadapted cells to a density of 3.2×10^{4} cells/ml

Amino acid	Without *dl*-allyl glycine, cell count/ml	With *dl*-allyl glycine, cell count/ml
dl-Cysteine	3.0×10^{5}	2.8×10^{5}
dl-Methionine	1.3×10^{6}	5.2×10^{5}
dl-Homocysteine	5.0×10^{5}	1.2×10^{5}
None	1.5×10^{7}	5.8×10^{4}

comparisons were made between the level of growth occurring in control cultures with those obtainable in cultures containing 5×10^{-3} *M* concentrations of each of the amino acids in media supplemented or unsupplemented with 5×10^{-3} *M* allyl glycine. The results summarized in Table III show that, even in the absence of allyl glycine, 5×10^{-3} *M* concentrations of cysteine, homocysteine, or methionine are sufficiently toxic to suppress growth well below the control value. However, they also establish that cysteine alone permits as high a level of growth in the presence of allyl glycine as in its absence.

Thus, allowing for its intrinsic toxicity, only cysteine is capable of relieving completely the growth inhibition ascribable to allyl glycine. The partial effectiveness of homocysteine and methionine can be presumed to result from their abilities to spare cysteine.

From Table II it can be seen that the induction of respiratory variants by 5×10^{-3} *M* allyl glycine is suppressed completely by cysteine in concentration as low as 1×10^{-3} *M*. Serine in concentrations ranging from 1×10^{-3} *M* to 2×10^{-2} *M* is without effect. Since cysteine carbon is derived directly from serine, the mutagenicity of allyl glycine cannot be ascribed to interference with the generation of the cysteine carbon skeleton. On the other hand, the ability of allyl glycine to induce respiratory variants is conditioned markedly by those amino acids involved in the transfer of cysteine sulphur. Homocysteine, methionine, and the methionine derivative, alpha-amino-*n*-butyric acid, in concentrations of 2×10^{-3} *M*, 5×10^{-3} *M*, and 2×10^{-2} *M*, respectively, totally repress mutation. Conversely, homoserine in concentrations above 2×10^{-3} *M enhances* the mutagenicity of allyl glycine. This effect is a true potentiation of allyl glycine activity as homoserine alone in concentrations as high as 2×10^{-2} *M* is incapable of inducing respiratory variants. It is, furthermore, specific to homoserine since the homoserine derivative, threonine, does not influence the mutagenicity of allyl glycine. These findings indicate that there exists a particular metabolic function requiring cysteine which is essential to the genetic continuity of the aerobic respiratory mechanism of *Saccharomyces*. In cells unadapted to aerobic respiration that function is so limited that it can be disturbed selectively by growth in the presence of the cysteine antagonist, allyl glycine. Alpha-amino-*n*-butyric acid, homocysteine, and methionine probably alleviate this disturbance by their sparing action toward cysteine. Such an interpretation is supported by the fact that the comparative effectiveness of these amino acids in suppressing mutation corresponds to their relative placement in the biosynthetic sequence leading to cysteine (i.e., in order of increasing effectiveness: alpha-amino-*n*-butyric acid ⟶ methionine ⟶ homocysteine ⟶ cysteine). In these terms homoserine in high concentrations might accentuate the mutagenicity of allyl glycine by serving as a trap for cysteine via the formation of cystathione.

Discussion

While speculating upon the origin of cancer cells, Warburg proposed that the genetic continuity of the cellular aerobic respiratory mechanism requires the patterned generation of high-energy compounds within the chondriosomes (10). Since such activity occurs during aerobic but not fermentative respiration, he suggested that the growth of cells under anaerobiosis would be sufficient cause for the irreversible inactivation of their ability to respire aerobically. Lindegren and Hino (4) purport to have verified this hypothesis by demonstrating that anaerobiosis will induce respiration-deficient mutants in a haploid yeast culture. On the other hand, Harris (2) has concluded from an analogous but more comprehensive study employing a diploid yeast that

such induction does not occur. To explain this discrepancy Hino and Lindegren (3) have suggested that the production of respiratory mutants through anaerobic growth might be a ploidy-dependent phenomenon occurring only in haploid cells. However, investigations in our own laboratory upon a representative number of haploid as well as diploid, triploid, and tetraploid yeast strains have given no indication of a mutagenicity of anaerobiosis per se (5). They have demonstrated, though, that yeast cells of all ploidy classes are uniquely *predisposed* to loss of the potential for aerobic respiration when cultivated anaerobically. We have reported previously that respiration-deficient mutants are induced more readily by ultraviolet light in anaerobically than in aerobically grown cells (6). Unpublished findings have indicated that a similar situation obtains for inductions by acridine dyes or manganous ions. The results presented here have shown that anaerobic growth imposes upon cells some disturbance in the metabolism of cysteine which lessens their ability to preserve the capacity for aerobic respiration. The disturbance does not appear to involve a process for which the reducing properties of cysteine are essential since it arises during anaerobic growth and is manifested most severely in cells exposed anaerobically to the cysteine antagonist, allyl glycine. The abilities of amino acids participating in sulphur metabolism to influence the induction of respiratory deficiency by allyl glycine suggest that the disorder centers critically about some function relating to cysteine sulphur. Though the precise nature of this defect remains to be elucidated, the present findings do substantiate Warburg's essential insights to the extent that they establish the existence in the yeast cell of a metabolic system specifically concerned with the stability of the genetic determinant of respiration which is debilitated when cells are grown under anaerobiosis.

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EFFECT OF ACID HYDROLYZATES OF AGAR ON THE GROWTH OF ESCHERICHIA COLI[1]

W. YAPHE

Abstract

Studies were carried out to determine the effect of the products of acid hydrolysis of agar on the growth of *Escherichia coli*. The inhibitory effect was associated with the degree of hydrolysis of the agar. This inhibition has been obtained at two very different levels of concentration. When the hydrolysis was complete, the inhibitory pattern was similar to that of 5-hydroxymethyl-2-furaldehyde. Under conditions of mild acid hydrolysis, the inhibitory effect was caused by a heat-labile degradation product of 3,6-anhydro-L-galactose.

Introduction

Preparations sold as agar for bacteriological purposes contain a number of different polysaccharides (8). In this communication, agar is considered to be any preparation which contains agarose. Agarose consists essentially of linear chains of D-galacto-pyranose residues, linked through positions 1 and 3, and 3,6-anhydro-L-galactopyranose residues, linked through positions 1 and 4 (1). The 3,6-anhydro-L-galactopyranosidic linkages are very labile to acid, and are readily hydrolyzed with the formation of a reducing end group of 3,6-anhydro-L-galactose. This is the reason for the loss in gel strength and increase in reducing power of preparations of agar which have been autoclaved for too long a period. Under mild conditions of acid hydrolysis, any free 3,6-anhydro galactose will undergo dehydration to form 5-hydroxymethyl-2-furaldehyde (HMF).

Commercial agar has been shown to contain substances which will inhibit (3), stimulate (5), or act as nutrients for the growth of different microorganisms. Robbins and McVeigh (6) observed that the growth of *Escherichia coli* was inhibited by an agar solution which had been hydrolyzed with 0.1 *N* H_2SO_4. The products of partially hydrolyzed agar are as follows: agarose → oligosaccharides → agarobiose → galactose + 3,6-anhydro galactose → dehydration products of 3,6-anhydro galactose → HMF → formic + levulinic acids. Galactose and HMF are the products after complete acid hydrolysis of agar. The object of the present investigation was to determine the effect of the products of acid hydrolysis of agar on the growth of *E. coli*.

Materials and Methods

Commercial agar (Difco) and a purified preparation (8) of this agar were used in the experiments. The agar was dissolved in various normalities of oxalic acid and heated in an autoclave at 121° C. The solution was neutralized with solid $CaCO_3$, centrifuged, and passed through a Seitz filter.

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HMF was prepared from sucrose and agar by the method of Haworth and Jones (2). The HMF concentration of the agar hydrolyzates was calculated from the molar extinction coefficient of HMF, 16,500, at 285 mμ (7). The optical densities and ultraviolet absorption spectra of the solutions were determined with a Beckman DU spectrophotometer.

The chromatographic techniques and spray reagents used to identify the sugars were previously described (8).

The strain of *E. coli* was obtained from Camp Hill Hospital, Halifax, N.S. The basal medium contained the following, per liter of distilled water: K_2HPO_4, 7.0 g; KH_2PO_4, 3.0 g; $MgSO_4.7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1.0 g; and bromocresol purple, 1 ml of a 1.6% solution. A solution of lactose, 20 g per 100 ml, was sterilized by filtration using a Seitz filter and was added to the basal medium in the ratio of 1:9.

The effect of the agar hydrolyzate on the growth of *E. coli* was determined by the tube dilution method. The test was performed in 2-ml amounts. One milliliter of an 18-hour culture in nutrient broth (Difco), containing 10^8 bacteria per milliliter, was added to 100 ml of the basal medium. One milliliter of the inoculated medium was then added to a series of tubes which contained varying amounts of the agar hydrolyzate in 1 ml of distilled water. A growth control tube which contained 1 ml of inoculum and 1 ml of distilled water was used in each test. The tubes were incubated at 37° C and growth was recorded after 18, 24, and 48 hours. The inhibitory concentration was taken as that in the first culture tube to show absence of growth.

For the disk-plate assay, 0.1 ml of the test substance was added to 12.7 mm Schleicher and Schuell paper-disks. The disks were placed on the surface of agar previously seeded with *E. coli* and the zone of inhibition was measured after 24 hours at 37° C.

Results and Discussion

Effect of Agar Concentration

The ultraviolet absorption spectra of the agar hydrolyzates were similar to that of HMF (Fig. 1). The results (Table I) show that in the hydrolyzates where the concentration of HMF varied from 0.48 to 4.07 mg/ml, the growth of *E. coli* was inhibited at 0.75 to 1.00 mg of the hydrolyzate which contained 65 to 95 μg of HMF. *E. coli* was not inhibited by the 0.1% agar hydrolyzate which contained 0.5 mg of hydrolyzate and 44 μg of HMF per milliliter of basal medium.

Pure HMF which was prepared from agar and sucrose inhibited the growth of *E. coli* at a concentration of 2100 μg per milliliter of growth medium. Therefore under the conditions of partial acid hydrolysis, the inhibitory effect of the acid hydrolyzate was not due to HMF but must have been caused by some other product, the concentration of which was proportional to that of HMF.

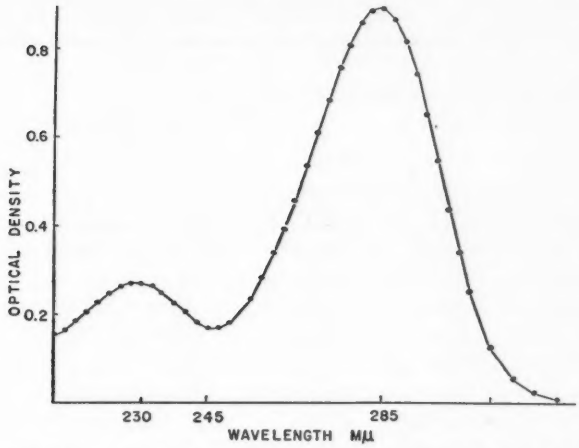

FIG. 1. The ultraviolet absorption spectrum of a 5% solution of washed agar in 0.01 *N* oxalic acid after heating at 121° C for 1 hour.

TABLE I

Concentrations of the degradation products of agar inhibitory to the growth of *E. coli*. Washed agar, dissolved in 0.01 *N* oxalic acid and heated at 121° C for 2.25 hours

Initial concn. of agar, %	Concn. of HMF in hydrolyzate, mg/ml	Minimal inhibitory concn. after incubation periods of: 18 hr Agar hydrolyzate, mg/ml	18 hr HMF, μg/ml	48 hr Agar hydrolyzate, mg/ml	48 hr HMF μg/ml
0.1	0.09	*	*	*	*
0.5	0.48	1.00	95	1.34	130
1.0	0.93	0.80	75	1.30	125
1.5	1.46	0.75	75	1.22	130
3.0	2.71	0.75	70	1.50	135
5.0	4.07	0.80	65	1.65	135

* No inhibition.

Effect of Partial and Complete Acid Hydrolysis

The results (Table II) show that as the heating time was increased from 0.5 to 11 hours, the HMF concentration in the hydrolyzate increased from 0.28 to 9.90 mg per milliliter. At the 48-hour incubation period, the growth of *E. coli* was inhibited by 2.00 to 10.00 mg of the hydrolyzates which contained 26 to 1980 μg of HMF.

Similar results were obtained when the acid concentration was increased from 0.001 *N* to 0.16 *N* oxalic acid. With these hydrolyzates the growth of *E. coli* at the 48-hour period was inhibited by 1.25 to 11.25 mg of hydrolyzate which contained 80 to 1300 μg of HMF (Table III). These results indicate that on complete acid hydrolysis of agar, the inhibitory effect is due to HMF.

TABLE II

Concentrations of the degradation products of agar inhibitory to the growth of *E. coli*. A 5% solution of agar in 0.02 *N* oxalic heated at 121° C

Time of hydrolysis, hr	Concn. of HMF in hydrolyzate, mg/ml	Minimal inhibitory concn. after incubation periods of: 18 hr — Agar hydrolyzate, mg/ml	18 hr — HMF, μg/ml	48 hr — Agar hydrolyzate, mg/ml	48 hr — HMF, μg/ml
0.5	0.28	5.00	30	5.00	30
1	0.59	1.67	17	2.50	26
2.25	3.20	1.25	80	2.00	125
3.5	6.02	1.13	135	—	—
5	7.52	3.50	525	3.75	560
8	8.43	7.50	1250	10.00	1680
11	9.90	7.50	1485	10.00	1980

TABLE III

Concentrations of the degradation products of agar inhibitory to the growth of *E. coli*. A 5% solution of washed agar heated at 121° C for 2.25 hours

Concn. of oxalic acid, *N*	Concn. of HMF in hydrolyzate, mg/ml	Minimal inhibitory concn. after incubation periods of: 18 hr — Agar hydrolyzate, mg/ml	18 hr — HMF, μg/ml	48 hr — Agar hydrolyzate, mg/ml	48 hr — HMF, μg/ml
0.001	0.44	6.25	55	7.75	75
0.005	2.03	1.43	60	2.14	90
0.01	3.22	1.00	65	1.25	80
0.02	4.49	1.34	110	1.50	134
0.04	5.61	3.00	330	3.50	390
0.08	6.63	3.13	415	4.50	600
0.16	—	5.00	580	11.25	1300

With an agar hydrolyzate which had been incubated for 15 days at 37° C, the growth of *E. coli* was inhibited by 16.26 mg of the acid hydrolyzate which contained 400 μg of HMF as compared with 1.0 mg of hydrolyzate which contained 70 μg of HMF for the control solution. This leads to the conclusion that the unidentified inhibitory substance in the partial hydrolyzate of agar was heat labile.

E. coli was able to grow in the basal medium to which was added a 4% solution of an enzyme hydrolyzate of agar (8). This indicates that the commercial preparation of agar did not contain substances which would inhibit the growth of this organism.

Five per cent hydrolyzates of galactose and xylose did not inhibit growth, which indicates that the inhibitory effect obtained with the agar hydrolyzates was not caused by products of acid hydrolysis of the galactose moiety of agar or by the xylan which is present in commercial agar.

The growth of *E. coli* was not inhibited by a preparation of 3,6-anhydro galactose which had been obtained from a carbon column to which a 5% acid hydrolyzate of agar had been added.

Growth was not inhibited in the basal medium to which was added solutions of 0.1 *N* levulinic and formic acid.

Products of Acid Hydrolysis

Chromatograms of the acid hydrolyzates were sprayed with aniline hydrogen phthalate and a modified Seliwanoff reagent (8). The chromatograms of the hydrolyzates described in Tables II and III showed that with increasing hydrolysis the concentration of galactose and HMF increased while the concentration of 3,6-anhydro galactose and products of degradation of 3,6-anhydro galactose decreased. In spite of streaking, the chromatograms could be divided into seven fractions: (1) oligosaccharides; (2) galactose; (3) xylose; (4) agarobiose; (5) 3,6-anhydro galactose; (6) products of degradation of 3,6-anhydro galactose; (7) HMF. Fractions 1 to 7 were eluted from sheets of Whatman No. 3 filter paper. With disk-plate method of assay, an inhibitory effect was observed with fractions 6 and 7. Similar results were obtained when the density of the spots on the chromatograms, as determined according to Martin (4), was correlated with the inhibitory pattern of the hydrolyzate.

The results indicated that the inhibitory effect of acid hydrolyzates of agar on the growth of *E. coli* was due to 5-hydroxymethyl-2-furaldehyde and an unidentified heat-labile component. Under conditions of mild acid hydrolysis the effect was caused by the latter compound, while on complete acid hydrolysis the inhibition was due to the former. The chromatographic evidence suggests that the unidentified component is a degradation product of 3,6-anhydro-L-galactose.

Acknowledgments

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THE AGE FACTOR IN EXPERIMENTAL COXSACKIE B-3 INFECTION[1]

E. Irene Grodums and George Dempster

Abstract

A detailed study of the influence of the age factor upon the susceptibility of the white mouse to experimental infection with a standard strain of Coxsackie B-3 virus has been undertaken. A fairly accurate assessment of the susceptibility of the brain, heart, and brown fat tissues has been achieved by examining sufficiently large groups of animals inoculated at ages varying from 4 days to 182 days by histological and virological procedures.

The contrasting patterns of changing susceptibility in the brain and heart were quite remarkable. Brain lesions were not found in mice inoculated after 12 days of age but heart lesions were severest in animals inoculated between 12 and 23 days of age. In both heart and brown fat tissue lesions could be found in adult animals infected with Coxsackie B-3 virus. Attention is drawn to the fact that the pathological response in the brown fat tissue is different in sucklings, weanlings, and adult mice.

Introduction

The present communication is the first in a series of investigations devoted to a study of the role of lipids in the growth and pathogenesis of neurotropic viruses. The group B Coxsackie viruses were selected because of their peculiar trophism for the brown fat tissue (8, 20, 21, 36). It was felt that a thorough investigation of this characteristic by a variety of techniques was something which had not yet been attempted and should provide a more fundamental understanding of the part played by lipids in virus diseases of man and animals. Furthermore, since the Coxsackie B viruses are also neurotropic (9, 12, 16–20, 24, 26–28, 36, 38) the part played by different lipids present in brain and brown fat could be assessed and any possible interrelationship between the two very different tissues could be studied.

As a first step in the systematic investigation of the pathogenesis of Coxsackie B virus infection it was decided to examine the virus–host relationship between a known standard mouse-adapted B-3 virus strain and an inbred strain of albino mice. In the following experiments attempts were made to keep all other factors constant and only the age of the experimental host was varied. The virus strain, the route of inoculation, the passage material used, and the time following inoculation of previously randomized mice were all kept constant.

In the past, experiments with large numbers of animals have usually been designed to elucidate several different aspects of the pathogenesis of the disease at one time. This has resulted in the histological examination of small groups of animals under varying circumstances (25, 29–32, 34–36, 40, 42). In this way, Pappenheimer *et al.* (36) reported upon five different

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strains when the viruses were in states of passage varying from the original throat washings to brain suspensions of mouse-adapted virus. Melnick and Godman (34) confined their investigations to one strain of Coxsackie B virus (Conn. 5) and sacrificed mice for histological examination at different times up to 14 days after inoculation. They limited their study to animals inoculated at either 1 day or 4–5 days of age, and used a mouse-adapted strain. Godman *et al.* (21) studied the same strain, type B_1 (Conn. 5), also in mice not older than 4–5 days and inoculated by the subcutaneous route.

The investigation of large groups of *adult* mice has been conducted mainly to demonstrate the influence of abnormal pharmacological or physiological stresses upon the outcome of the experimental disease. In this fashion the effects of cortisone and cold have been examined but the histological investigations have been limited to groups of 3–5 animals (2, 3, 31, 32). Other studies have been carried out with pancreatropic strains in adult mice (14, 22, 23, 37, 39).

It was hoped that by using larger groups for both histological and virological examination and by limiting the number of variables, while at the same time extending the range of the known variable, i.e. age, from day-old mice to 6-month-old mice, significant changes in tissue susceptibility might be more clearly demonstrated.

The aim of the present work is to reveal the changing susceptibilities of brain, heart, and brown fat tissues in suckling, weaned, and adult mice.

Materials and Methods

Mice

These were albino mice from an inbred colony maintained in our own laboratories but originally derived from the Connaught Medical Research Laboratories. Mice of the same age were pooled and redistributed in groups by stratified random sampling. Twelve different age groups covering the age range from 4 days to 182 days were studied. A standard diet was maintained for the adult and weaned mice throughout the experiments.

Virus

The group B type 3 Coxsackie virus used was mouse-adapted and had the following history. The strain was isolated by Dr. Joseph Melnick and sent to Dr. Dalldorf's laboratory in Albany in October 1950, labelled Nancy M_2 10/20/50. In Albany the mouse carcass was passed intracerebrally for one passage. Subsequent passages were made with brain suspension, M_4, by the intraperitoneal route, and the remaining passages subcutaneously. The strain was received in our laboratory labelled Coxsackie group B type 3 (Nancy No. 50531, Gen 17) mouse brain. It has been given three further passages with mouse carcass as inoculum by the intracerebral route. The stock has a LD_{50} in newborn mice by the intracerebral route of $10^{-6.5}$. A standard inoculum of 0.03 ml of a 1:100 dilution of mouse carcass suspension was used in all experimental procedures.

Plan of Investigation

Mice of each age group were inoculated intracerebrally and 10 mice were set aside in each group for every experimental procedure. Thus where both histological and virological data were required at least 20 mice were used. The animals were observed twice daily for signs of infection and were killed on the seventh day after inoculation. Deaths occurring within 48 hours were considered as non-specific. The animals were killed by ether anaesthesia on the seventh day[2] and one half of each group was prepared for histological examination while the remainder were subjected to virological study.

Histological Examination

The suckling mice were prepared for histological study as described by Pappenheimer (36). In weaned and adult mice the organs were removed, examined macroscopically, then fixed, and embedded in paraffin. The blocks from all mice were cut in 8 μ or 4 μ thick sections and every 30th section was mounted. The sections were stained with haematoxylin and eosin, Heidenhain's haematoxylin, Luxol-Schiff's, and v. Kossa's stains (4).

Scoring of Lesions

The lesions were graded on a one- to four-plus scale: thus, + for occasional small lesions, ++ for several small lesions, +++ for frequently occurring more severe lesions, and ++++ when most of the organ tissues had undergone pathological change. Finally susceptibility was graded on a percentile basis using the same formula as Kilbourne $\{(n\times1) + (n\times2)\}/(\text{total } n\times4)$ etc. when $n\times4$ = maximum possible score. Percentage expression makes possible comparisons of scores in groups of varying size (33).

Virological Studies

Organs were removed with aseptic precautions, ground with sterile alundum powder, and diluted to a 20% suspension in Hanks balanced salt solution (B.S.S.). The suspension was centrifuged at 1000 r.p.m. for 10 minutes. The supernate was removed and stored at $-20°$ C to $-30°$ C.

In selected age groups an extra 10 mice were inoculated and were bled from the heart on the seventh day. The blood was ground up with alundum and tested as a 20% suspension in B.S.S. for presence of virus. Penicillin 250 units/cc and streptomycin 250 μg/cc were added to all organ and blood suspensions.

Tests for virus were performed in human kidney[3] tissue cultures. Tube cultures were grown in 20% human serum in medium 1066.[4] The cultures were washed thrice with 2% calf serum in B.S.S. and maintained in 1 ml of 2% calf serum in 1066. An inoculum of 0.1 ml of tissue suspension supernate was used. Three tubes were inoculated per specimen and were examined for 7 days. Cytopathogenic effects were checked by repeated (two or three) passage and subsequent neutralization testing with specific B_3 rabbit antiserum.

[2]Eight mice in the 4-day group and six mice in the 7-day group were too sick to survive the whole period of observation and were killed on the fifth or sixth day.

[3]Chang's strain of human kidney cell.

[4]Obtained from the Connaught Medical Research Laboratories.

Results

Signs of Infection

Overt signs of disease indicating severe impairment of the central nervous system appeared in almost all mice inoculated at the age of 1 day and 4 days. One third of the mice inoculated at 7 days of age showed no clinical signs and very few indeed appeared ill when inoculated as 9-day-old mice. The observed differences are shown in Fig. 1.

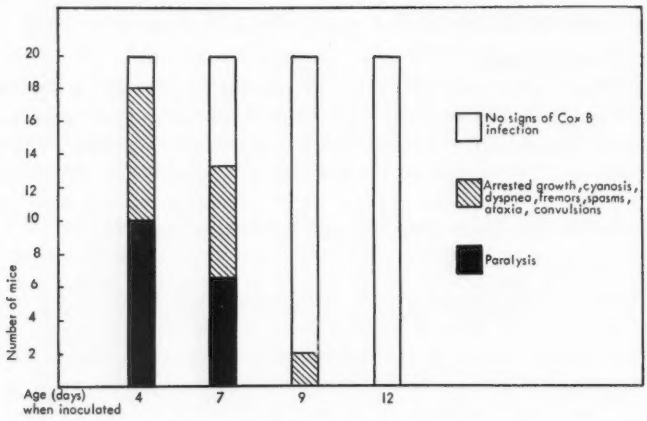

FIG. 1. Signs of Coxsackie B infection.

The Macroscopic Changes in the Brain, Heart, and Interscapular Brown Fat

The most dramatic changes were observed in the *brains* of mice inoculated up to the age of 7 days. Hydrocephalus of noticeable degree, and in many of marked degree (Fig. 2), was present in 16 out of 20 brains of the 4-day-old group and in 6 out of 18 brains of the 7-day-old group. Macroscopic changes were not observed in the brains of older age groups of mice.

In contrast the *hearts* of the older age groups (9 day and older) of mice on several occasions were covered with greyish-white patches which had a shrivelled appearance.

The interscapular *brown fat* pads of mice inoculated at various ages during the first 28 days of life had a whitish-creamy color and a brittle soap-like consistency. In contrast the brown fat pads of mice inoculated at a later age had become darker in color and had acquired a tougher, stringier consistency. Quite often, particularly in the adult males, the brown fat pads were very small.

FIG. 2. The hydrocephalic cerebrum of a mouse inoculated with Coxsackie B-3 when 4 days old, killed 7 days later, H.E. 15×

FIG. 3. A lesion in the cerebrum of a mouse inoculated with Coxsackie B-3 when 4 days old, killed 7 days later, H.E. 44×

FIG. 4. Magnification of Fig. 2. H.E. 300×

FIG. 5. Myocarditis in a mouse inoculated with Coxsackie B-3 when 63 to 70 days old, killed 7 days later, H.E. 44×

PLATE I

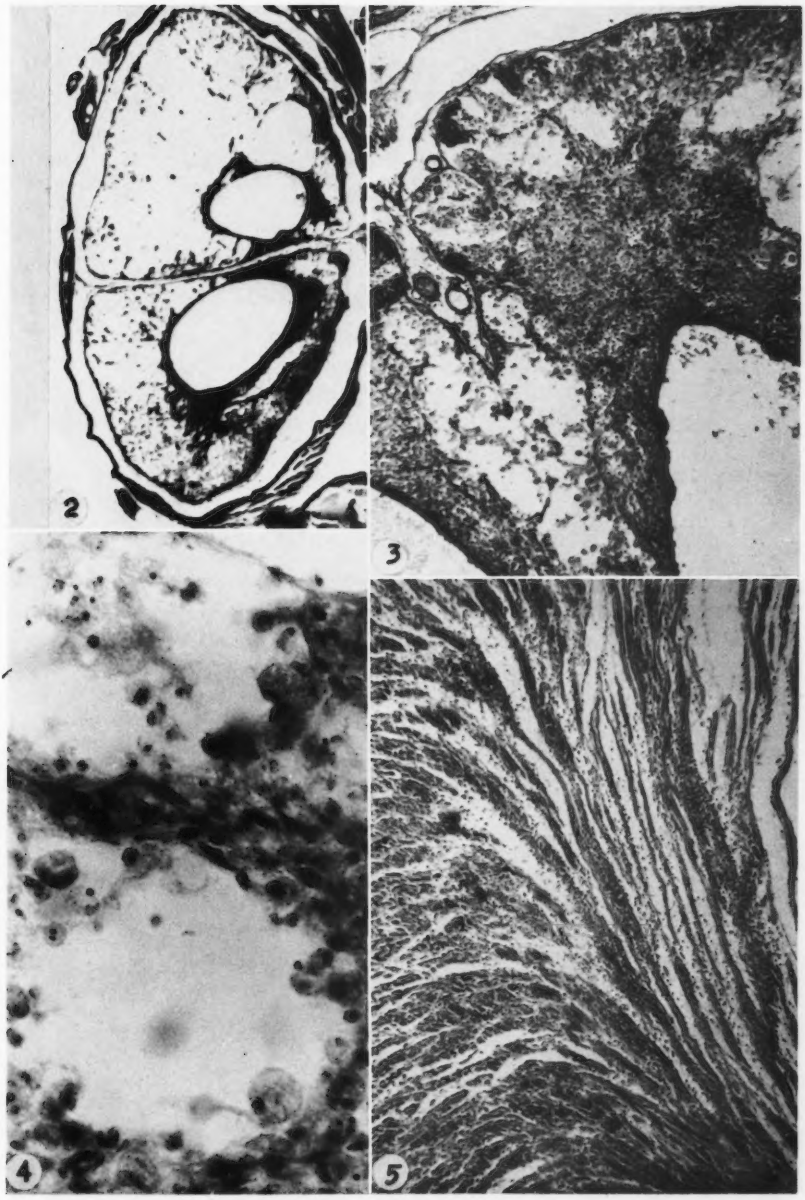

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PLATE II

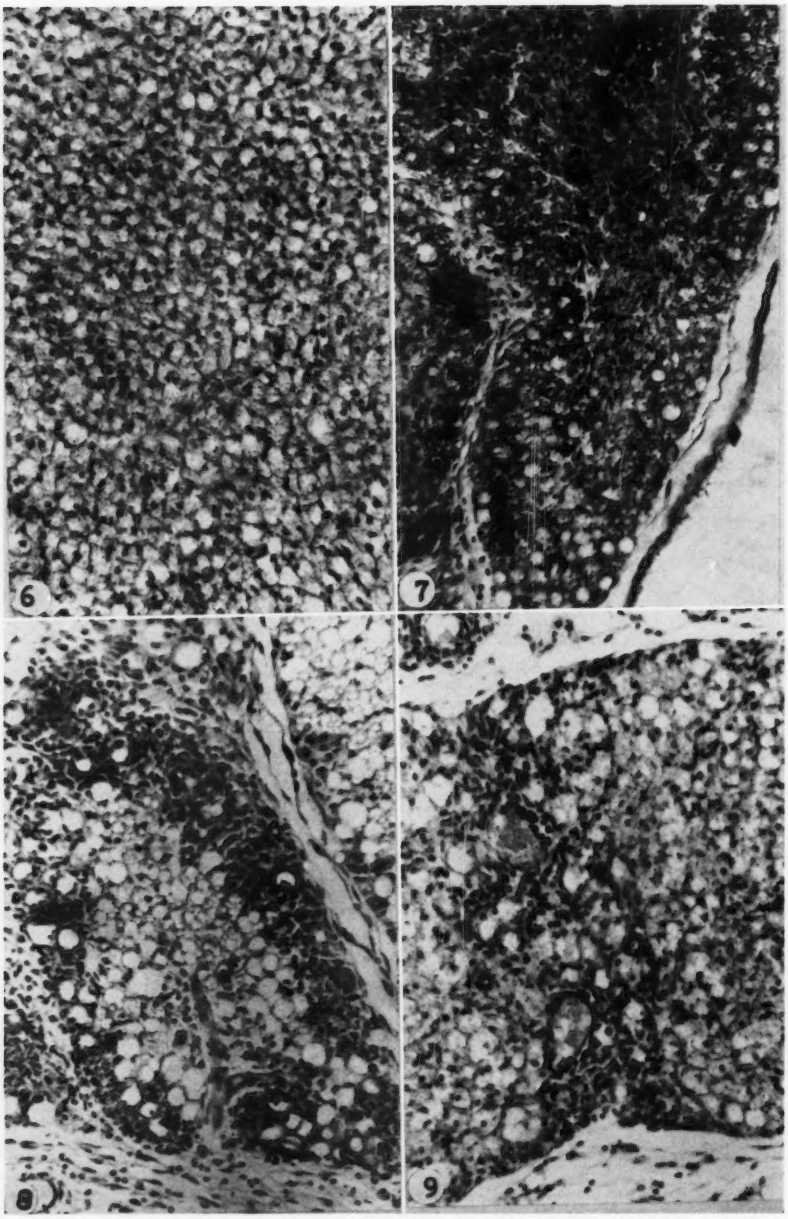

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TABLE I

The relative frequency of the histological lesion and the recovery of virus in mice inoculated at different ages

Age, days	Brain		Brown fat pads		Heart		Blood, Cox B-3	Marked clinical signs of disease‡
	Lesions*	Cox B-3†	Lesions*	Cox B-3†	Lesions*	Cox B-3†		
4	10/10	9/9	10/10	9/9	0/8	8/9	8/9	16/20
7	6/9	9/9	9/9	9/9	6/9	8/9	3/10	12/18
9	8/10	9/10	10/10	10/10	7/10	10/10	0/10	2/20
12	6/9	6/10	9/9	9/10	9/9	10/10	0/8	0/19
15	0/10	7/9	9/9	9/9	9/9	9/9	0/10	0/19
17	0/10	6/10	10/10	9/10	10/10	10/10		0/20
23	0/10	9/10	10/10	8/10	10/10	10/10		0/20
26	0/15	7/15	15/15	12/15	15/15	13/15		0/30
34	0/10	5/10	10/10	2/10	10/10	10/10		0/20
49–56	0/10	7/9	8/10	1/9	10/10	7/9	0/9	0/19
63–70	0/20	7/19	17/20	2/19	14/20	13/19		0/39
182	0/10	4/9	6/10	3/9	9/10	4/8		0/19

*Proportion of organs which show histological lesions.
†Proportion of organs yielding virus by tissue culture techniques.
‡Paralysis, ataxia, tremor, spasm.

The Microscopic Changes in the Brain, Heart, and Interscapular Brown Fat Pads

Little change in the pathological nature of the microscopic lesions in the *brain* (Figs. 3, 4) and in the *heart* (Fig. 5) was noticed over the range of age groups in which they could be found, although changes in the frequency of lesions in these tissues were observed (Table I). The morphological changes in the *brown fat* tissue, however, appeared to be quite striking and varied noticeably in accordance with the age of the mouse at the time of inoculation.

When the appearance of the lesions in the interscapular brown fat pads was taken as a basis for subdivision the inoculated mice could be separated into three distinct groups (Figs. 6–9).

Group I (newborn to 12-day-old mice)

(*a*) The periphery of the lobules shows extensive necrosis and slight inflammatory response.

(*b*) The center of the lobules shows a mixture of

(i) normal cells,

(ii) cells with *decreased* lipid globules,

(iii) granulation tissue.

In this group cells with decreased lipid globules predominate and normal cells are seldom encountered.

FIG. 6. Brown fat of a normal, uninoculated mouse, H.E. 125×

FIG. 7. Brown fat of a mouse inoculated with Coxsackie B-3 when 4 days old, killed 7 days later; note: the periphery of the lobule—necrotic zone; the center—cells with decreased lipid globules, H.E. 125×

FIG. 8. Brown fat of a mouse inoculated when 23 days old, killed 7 days later; note: the periphery of the lobule—necrotic zone; the center—cells with increased lipid globules, H.E. 125×

FIG. 9. Brown fat of a mouse inoculated when 182 days old, killed 7 days later; note: the periphery of the lobule—no necrosis; the center—cells in the center and the periphery of the lobule look alike, H.E. 125×

Group II (17-day-old to 26-day-old mice)

(*a*) The periphery of the lobules shows extensive necrosis and slight inflammatory response.

(*b*) The center of the lobules shows a mixture of

(i) normal cells,

(ii) cells with *enlarged* lipid globules,

(iii) necrotic cells with pycnotic nuclei and indistinct cell outline.

In this group the cells with enlarged lipid globules and the necrotic cells predominate equally.

Group III (34-day-old to 182-day-old mice)

(*a*) The periphery of the lobules shows changes which are identical with those found in the center.

(*b*) The center of the lobules shows a mixture of

(i) normal cells,

(ii) cells with enlarged lipid globules, some of which resemble white fat cells,

(iii) necrotic cells.

In this group there is great variation in the proportion of cells involved but a distinguishing feature is the total absence of peripheral necrosis.

It was interesting to note that the 15-day-old group held an intermediate position and in these animals the cells in some lobules showed the smaller lipid globules characteristic of group I, while in other lobules the enlarged lipid globules noted in group II were predominant.

The Susceptibility of the Brain, Heart, and Interscapular Brown Fat Pads

The relative frequencies of the presence of a histological lesion and the recovery of virus from the three tissues examined is shown in Table I. The possibility of viraemia existing as late as the time of examination of the organs (i.e. until 7 days) has been examined. It can be seen that a viraemic condition may exist in the mice inoculated in the 4-day-old and the 7-day-old groups. However, in older age groups this did not occur. Recovery of virus from the tissues of the older age groups, therefore, could not be attributed to the possibility of their being contaminated with blood containing the virus.

The following points illustrated by Table I seem to be worthy of note: (1) the persistence of Coxsackie virus in the *brain* tissue of mice inoculated at an age beyond which microscopic lesions could be demonstrated, (2) the finding of lesions in the *hearts* of older mice both weaned and adult, (3) the finding of lesions in the *brown fat* pads of weaned and adult mice, (4) the recovery of virus 7 days after inoculation from *all three tissues* in a significant proportion of animals inoculated at ages varying from 4 days to 6 months.

A full assessment of the susceptibility of the different tissues has to take into account not only the frequency of the presence of histological lesions but also the extent and severity of the lesions in the particular organ affected. The completeness of the histological examination (see Methods) enabled a fairly accurate assessment of these various considerations.

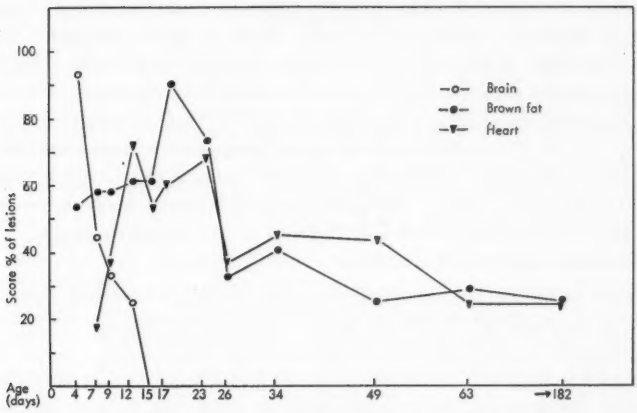

FIG. 10. The susceptibility of the brain, brown fat, and heart to Coxsackie B-3.

A distinct pattern of susceptibility is revealed. During the first 2 weeks of life the susceptibility of the brain declines rapidly *while at the same* time that of the heart rises steeply. Throughout this period of life the brown fat exhibits a steady degree of moderately severe susceptibility. In the third and fourth weeks of life the heart and brown fat are most highly susceptible and the brain shows no lesions whatever (Fig. 10).

Finally at the end of the fourth week of life (26 days) there occurs a drop in the susceptibility of the heart and brown fat, but both of these tissues continue to exhibit a very similar degree of moderate susceptibility right on into adult life (Fig. 10).

Discussion

It is possible from the experimental data presented to obtain a fairly accurate appreciation of the changing susceptibility of the different tissues examined. Biologically the host may be considered to be passing through four stages of development: the suckling, the weanling, the weaned, and the adult. Solid food is not eaten before 12–14 days (41) and the animals at this stage may be truly defined as sucklings. The length of the weanling stage is dependent upon the breeder and in the present experiments this stage was terminated at 28 days by removing the young mice from their mother's cage. Most writers in the literature have referred to mice of 4–6 weeks as being adult (2, 3, 43). However, for the purposes of the present discussion it would be better not to consider the animal adult until it has reached an age when it is capable of fertile mating, and this happens between 9 and 10 weeks of age in the colony under study.

Pathogenicity for the suckling mouse is the main taxonomic feature of the Coxsackie virus group (5–7, 10, 11, 13, 14) and naturally one would expect to find the maximum damage to the various tissues in animals of this age. It

was, therefore, surprising to the present workers to find that the strain being investigated showed a pattern in some respects quite different from the expected. As mice of the older age groups were inoculated the susceptibility of the brain tissue steadily decreased and with this decrease obvious signs of Coxsackie group B infection diminished until in 15-day-old animals inoculations produced neither signs nor histological lesions of affection of the C.N.S. What was not expected, however, was the completely inverse pattern of response of the heart tissue. In the youngest animals there were no heart lesions and in animals inoculated at the end of the suckling period (12 days) the severest reactions were recorded.

The severest damage to the heart and the brown fat tissues was recorded in the weanling stage at a time when the brain, although it would support growth of the virus, showed no histological response to it.

Once weaning was well established in the third week of life there occurred a significant decrease in the susceptibility of both the heart and brown fat tissues. However, both of these tissues remained moderately severely affected when weaned animals were inoculated. Even with the onset of sexual maturity and adult existence at 9 weeks the heart and brown fat retained a moderate degree of susceptibility which was still demonstrable in the adult group tested at 6 months.

Since the purpose of the present series of investigations was to evaluate the effects of lipid substances upon the pathogenesis of Coxsackie group B infections, considerable interest was evinced in the three different types of response observed in the brown fat. The changes, although gradual, were distinct. Typical peripheral necrosis of the lobules of the brown fat was seen only in sucklings and weanlings and was entirely absent in the weaned and adult animals.

Amongst the other changes exhibited in the brown fat of animals of different ages were changes in the size of the intracellular droplets. This may be of considerable significance, for Deane, in her review of the significance of intracellular lipids, considers droplet size of prime importance in the assessment of cell activity and places only secondary emphasis upon the droplet activity as revealed by histochemical methods (15).

It is natural then to wonder whether the physiological or the pathological activity of the brown fat as an organ affects the susceptibility of other tissues. The data presented shows how closely the heart tissue follows the brown fat in the severity of its response. Further experiments are being designed to determine whether such an interrelationship exists between the brown fat and other tissues such as the heart. In this connection reference should be made to Aronson, Schwartzman, and Teodoru's work and the recovery of poliomyelitis virus from the brown fat of hamsters before it could be shown to be present in other tissues (1).

While it is tempting to suggest that a relationship may exist, the responses observed may equally well be the reflection of changes in the maturity of the tissues involved and dependent rather upon dietary or endocrinal change.

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MYOCARDITIS IN EXPERIMENTAL COXSACKIE B-3 INFECTION[1]

E. IRENE GRODUMS AND GEORGE DEMPSTER

Abstract

Myocarditis has been produced in both young and adult mice by the inoculation of Coxsackie B-3 virus. Newborn mice were not as susceptible to myocardial damage as 12-day-old mice and the susceptibility increased up to the age of 23 days; thereafter there was a decline but 6-month-old animals were moderately susceptible.

Both the strain of animal used and the age at which it was inoculated appeared to be significant factors. Other factors such as route of inoculation, sex, virus concentration, or nature of the inoculating material did not greatly affect the outcome. The lesions produced were very similar to those described in human myocarditis of the newborn.

The importance of these findings lies in the fact that myocarditis due to Coxsackie virus can be produced regularly in both young and adult mice without the aid of cortisone or other extrinsic factors.

Introduction

Heart lesions in experimental Coxsackie infections have received comparatively little attention in the past. With the discovery that the group B viruses are associated with a fatal myocarditis of the newborn of man (3, 7, 13, 14, 17, 18, 26, 28, 30, 31, 33) and may also be a cause of cardiac illness of the adult human patient (8, 9, 15, 24, 25, 29, 32, 35) the importance of the experimental disease is surely worthy of greater attention.

Perhaps one reason for this neglect has been the infrequent occurrence of myocarditis in the series of animals which have been examined. Dalldorf (6) comments upon the paucity of lesions in the heart, and his co-worker Gifford (10) when examining suckling mice infected with several different types of Coxsackie B virus found myocarditis only in 3 of 51 mice subjected to careful examination. Higher incidences in 4- to 5-day-old mice were recorded by Melnick (29%) (22) and by Godman (28%) (11) using the intraperitoneal and subcutaneous routes of inoculation respectively. Both of these workers employed the Conn. 5 strain of Coxsackie B, type 1.

In contrast to these observations Verlinde (33) found that myocarditis was a constant manifestation in suckling mice infected with Coxsackie B-4 in tissue suspensions from the heart muscle of infants who had died from histologically proved myocarditis. However, no actual figures of incidence are quoted.

Dalldorf (4) wrote that animals more than 12 days of age were resistant to infection. He noted also that the resistance of mature mice was not due to the peripheral barriers that interfere with certain other virus infections, for older mice were equally resistant to intracerebral inoculation of Coxsackie

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virus (5). Pappenheimer *et al.* used the Powers strain (27) of Coxsackie B virus for intracerebral inoculation of mice and observed that heart lesions did not occur in mice inoculated after the sixth day. Myocardial lesions, however, have been obtained in adult mice which have been exposed to cold shortly after inoculation (2, 34), or to cortisone treatment before inoculation with one of the Coxsackie group B viruses (1, 19–21).

It was of great interest, therefore, when the present authors found that they could regularly produce myocarditis (12) both in young and in adult mice with the host – Coxsackie virus system which they were employing to investigate the role of lipids in the pathogenesis of neurotropic viruses.

The present paper is an extension of this work with a particular strain Coxsackie B-3 virus. In particular, it is a study of the effects of various factors upon the frequency and severity of the myocardial lesion in mice from birth to adult life. Type B-3 virus was selected because of the ease with which it may be titrated in tissue culture and also because it has been reported as a cause of myocarditis in newborn infants (14, 17, 31).

Materials and Methods

Mice

In the present investigations mice were used from the following sources:

(1) from an inbred colony of albino mice (12);

(2) fawn-colored mice which have been derived by crossing the aforementioned strain of albino mice with a wild mouse;

(3) 63- to 70-day-old mice which were kindly supplied by the late Miss Sickles from the stock of the Division of Laboratories and Research, New York Department of Health, Albany.

Virus

The stock Coxsackie group B-3 strain in mouse carcass suspension which was used in previous experiments (12) was employed in these studies. This suspension had a LD_{50} in 1-day-old mice inoculated by the intracerebral route of $10^{-6.42}$. The titer by the subcutaneous route was $10^{-6.71}$. The same suspension produced a TCD_{50} of $10^{-5.25}$ per 0.1 ml in the supernate of human kidney cell cultures.

Tissue Culture

Continuous human kidney cell cultures were used for isolation of virus and for neutralization tests. The methods have been described in a previous report (12).

Histological Examination

The hearts were fixed in 4% formaldehyde solution, embedded in paraffin, and cut in 8 μ sections. Each 30th section was mounted and stained with haematoxylin and eosin.

Plan of Investigation

Randomized mice of various age groups (from 4- to 182-day-old) were inoculated by the intracerebral route and killed 7 days later (12). A standard

inoculum of 0.03 cc 10^{-2} Coxsackie B-3 in mouse carcass suspension was used throughout except when it was desired to observe the effects of using a tissue culture supernate which would be free from mouse carcass components. Tissue culture supernate was tested undiluted in 15-day-old and 34-day-old mice.

The route of inoculation was varied in the 4-day-old, 12-day-old, 17-day-old, and 63- to 70-day-old groups. Mice of the selected ages were divided into two groups, one group was inoculated by the intracerebral route, the other by the subcutaneous route just above the interscapular brown fat pad. Equal numbers of the inoculated mice were examined by histological and virological procedures. For the virological examination the hearts were pooled, ground, and the suspensions were tested for the presence of Coxsackie B-3 virus (12). The effects of crossing our own albino strain with a wild strain of mouse were tested in one of the most susceptible age groups, the 17-day-old group (12).

In the adult mouse group (63- to 70-day-old) various other factors were taken into consideration, such as virus dose, sex, length of survival time, and also host strain.

Controls

In a control series of tests the following groups of mice were examined by similar procedure:

(1) 63- to 70-day-old mice were inoculated with 100 LD_{50} Coxsackie B-3 mixed with an equal amount of the specific 1:5 Coxsackie B-3 hamster antiserum;

(2) normal uninoculated mice of corresponding ages;

(3) normal mice inoculated with normal mouse passaged carcass suspension (three passages by the intracerebral route).

Results

The Frequency and Severity of Myocardial Lesions

Subcutaneous Route

The frequency and severity of myocardial lesions caused by Coxsackie B-3 infection appeared to be similar when mice were inoculated by either the intracerebral or the subcutaneous route. The results are presented in Fig. 1. The ages have been selected so that they would represent two groups of mice with the lowest susceptibility—one from the younger (4-day-old), the other from the older age groups (63- to 70-day-old)—and two groups representing mice with the highest susceptibility (12- and 17-day-old) (12).

In subsequent experiments only the intracerebral route of inoculation was used.

Intracerebral Route

The frequency of the myocardial lesions, when mice of various ages were inoculated intracerebrally, have already been reported (12). It should be pointed out, however, that practically no lesions were found in the hearts

of mice inoculated up to the age of 4 days. Myocarditis was present in all mice when inoculated from the age of 12 to 56 days and the hearts of older mice, up to the age of 182 days, were affected in 70 to 90% of cases. The severest lesions were observed in mice between the ages of 12 and 23 days. The severity of the microscopic lesions have been evaluated as described earlier (12) and the results have been summarized in Table I. As a rule, macroscopic lesions could be observed in hearts which showed +++ and ++++ upon microscopic examination.

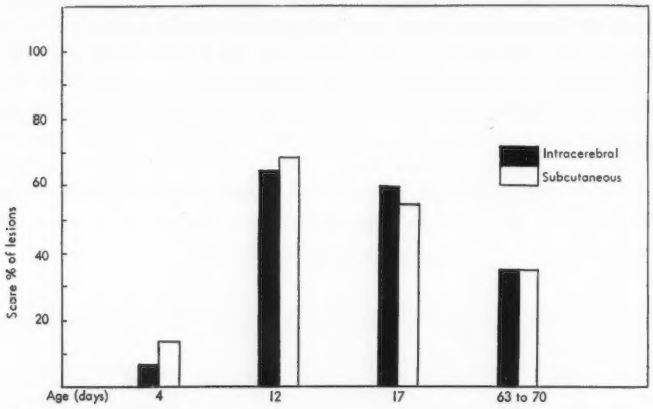

FIG. 1. Susceptibility of the myocardium to Coxsackie B-3. A comparison of two different routes of inoculation.

TABLE I

Frequency and severity of myocarditis in mice at various ages

Age (days) inocul.	Frequency and severity of myocarditis						Statist. signif., χ^2
	0	+	++	+++	++++	Totals	
				Part *a*			
4	8	0	0	0	0	8	
7	3	6	0	0	0	9	
9	3	5	2	0	0	10	
12	0	0	1	8	0	9	83.108*
15	0	2	3	3	0	8	
17	0	1	6	1	2	10	
23	0	0	4	5	1	10	
Totals	14	14	16	17	3	64	↓
				Part *b*			
23	0	0	4	5	1	10	↑
26	0	9	5	1	0	15	
34	0	3	6	1	0	10	
49–56	0	3	7	0	0	10	56.125*
63–70	6	10	3	1	0	20	
182	1	8	1	0	0	10	
Totals	7	33	26	8	1	75	

*Highly significant.

An analysis by statistical methods (16) confirms the significance of the increase in susceptibility of the myocardium (expressed as a function of the frequency and severity of the pathological lesions) up to the age of 23 days. The heart was affected less after the age of 23 days but was not free from lesions. In adult 182-day-old mice the myocardium was affected in 9 out of 10 animals inoculated. The pathological changes in the adult animals were not very severe but the score percentage recorded for this group was higher than the corresponding figure for the 4- and 9-day-old mice (Table II).

TABLE II

A comparison between the susceptibility of the myocardium to Coxsackie B-3 in the suckling and adult mice

Age (days) inocul.	Frequency of myocarditis	Frequency and severity of myocarditis					
		0	+	++	+++	++++	Score%*
4 to 9	13/27	14	11	2	0	0	14
63 to 182	23/30	7	18	4	1	0	24

*The score percentage recorded in this and subsequent tables was calculated using the formula $\{(n\times1) + (n\times2)\}/(\text{total } n\times4)$ etc. when $n\times4$ = maximal possible score. Percentage expression makes possible comparison of scores in groups of varying size (21).

Concentration of Virus

For this investigation sexually mature mice only were used (63- to 70-day-old.) It was reasoned that if Coxsackie B-3 virus would cause myocarditis in adult mice, one could assume that it would have a similar, or a more pronounced, effect in the more susceptible younger age groups. It was obvious from the results obtained that Coxsackie B-3 virus at a concentration of $10^{-4.5}$ was as effective as at a concentration of 10^{-2} (Table III). The mice were killed 7 days after inoculation.

TABLE III

Myocarditis of 63- to 70-day-old mice: the effects of the strength of the inoculum

Virus concn.	Frequency of myocarditis	Frequency and severity of myocarditis					
		0	+	++	+++	++++	Score%
10^{-2}	6/10	4	2	3	1	0	28
$10^{-4.5}$	9/10	1	6	3	0	0	30

The Time Factor

So far all the inoculated mice had been killed 7 days after inoculation except for the very sick animals, which had to be harvested earlier in the 4-day-old group. The question arose as to how the frequency and the severity of the myocardial lesions would change if the time of survival was increased. Again, only the adult mice were chosen because:

(1) they were the most likely ones to survive for an extended period of time;

(2) further development of the myocarditis would be easier to evaluate in this group since in the weaned mice, even 7 days after inoculation, most of the heart had undergone pathological changes.

The results showed that, as the time after the inoculation increased, the observed myocardial lesions were less frequent and less severe (Table IV).

TABLE IV

Extent of myocardial involvement in adult mice at various periods after inoculation

Killed, days after inocul.	Frequency of myocarditis	Frequency and severity of myocarditis					
		0	+	++	+++	++++	Score%
7	9/10	1	4	5	0	0	35
14	7/10	3	4	3	0	0	25
28	7/10	3	7	0	0	0	18

The Nature of the Inoculum

The Coxsackie B-3 virus was inoculated intracerebrally in mouse carcass suspension and in tissue culture supernate into 15- and 34-day-old mice. When the frequency and severity of the lesions were evaluated in 15-day-old mice a higher per cent score value was recorded if virus was inoculated as tissue culture supernate. The substitution of a tissue culture inoculum appeared to make some difference in 34-day-old mice but the effect was not as marked (Table V).

It would appear that the presence of mouse tissue in the inoculum does not have an enhancing effect upon the frequency and extent of the myocardial lesions: in fact, an inhibitory action may be at work.

TABLE V

Coxsackie B-3 inoculated in mouse carcass suspension and in tissue culture supernate

Age (days) inocul.	Inoculum	Virus concn.	Frequency of myocarditis	Frequency and severity of myocarditis					
				0	+	++	+++	++++	Score%
15	Mouse carcass	10^{-2}	8/8	0	2	3	3	0	53
15	Tissue culture supernate	Undilut.	7/7	0	0	2	5	0	68
34	Mouse carcass	10^{-2}	10/10	0	3	6	1	0	45
34	Tissue culture supernate	Undilut.	10/10	0	2	6	2	0	50

Strain of Mice

It was found that the cross (fawn) resulting from the stock albino mice and a wild mouse was just as susceptible as the original stock. The Albany strain in contrast were much less susceptible. Furthermore, the lesions in the latter strain were less extensive (Table VI).

TABLE VI

The susceptibility of the myocardium to Coxsackie B-3 in different strains of mice

Age (days) inocul.	Strain of mice	Frequency of myocarditis	Frequency and severity of myocarditis					
			0	+	++	+++	++++	Score%
17	Fawn	9/9	0	0	6	3	0	58
17	Stock	10/10	0	1	6	1	2	60
63–70	Albany	4/9	5	4	0	0	0	11
63–70	Stock	14/20	6	10	3	1	0	23

The Sex Factor in Adult Mice

The susceptibility of the myocardium to Coxsackie B-3 was tested separately in 63- to 70-day-old male and female mice. Although the susceptibility of the male mice appeared to be slightly higher than that of the females (Table VII), it was also obvious that myocarditis was a common feature for both sexes of adult mice inoculated with Coxsackie B-3 virus.

TABLE VII

Sex factor in myocarditis of 63- to 70-day-old mice

Sex	Frequency of myocarditis	Frequency and severity of myocarditis					
		0	+	++	+++	++++	Score%
Male	6/10	4	2	3	1	0	28
Female	8/10	2	8	0	0	0	20

Controls

1. Myocardial lesions were not observed in 63- to 70-day-old mice inoculated with 100 LD_{50} Coxsackie B-3 virus suspension mixed with equal parts of 1:5 of the specific Coxsackie B-3 hamster antiserum.

2. The hearts of 95 normal mice examined at various ages also appeared to be free of lesions.

3. The inoculation of *normal* passaged mouse carcass suspension (three passages by intracerebral route) did not cause any pathological changes in any of the organs in newborn or in 15-day-old mice. However, this tissue did stimulate the production of myocardial lesions in 63- to 70-day-old mice irrespective of the dilution used (Table VIII). This is a very interesting and puzzling feature which requires further investigation.

TABLE VIII

Frequency and severity of myocardial lesions in mice inoculated with normal passaged carcass suspension

Age (days) inocul.	Concn. of suspens.	Frequency of myocarditis	Frequency and severity of myocarditis					
			0	+	++	+++	++++	Score%
15	10^{-2}	0/6	6	0	0	0	0	0
63–70	10^{-2}	5/19	14	4	1	0	0	8
63–70	$10^{-4.3}$	2/10	8	2	0	0	0	5

Recovery of Virus

The Coxsackie B-3 virus could be recovered 7 days after inoculation in 100% of hearts of mice when inoculated intracerebrally at any age up to 56 days. This finding has been mentioned in a previous report (12). Suspensions made from pools of hearts of mice inoculated subcutaneously were tested similarly on continuous human kidney cell cultures. Coxsackie B-3 virus was recovered 7 days after inoculation from the pools of all age groups tested (4-, 12-, 17-, and 70-day-old). In addition virus was recovered both from the group of fawn mice which were inoculated when 17 days old and from the Albany mice which were inoculated when adult (63- to 70-day-old).

Virus was never demonstrated by tissue culture methods in the heart or blood of normal uninoculated mice, neither was it found in mice inoculated with thrice passaged normal mouse carcass.

Morphology of the Heart Lesions

Pathological changes in the hearts of the mice inoculated with the Coxsackie B-3 virus were found in the pericardium (Fig. 2) and in the myocardium (Figs. 3, 4, 5, 6, 7, and 8) but lesions were not observed in the endocardium.

The morphological character of the myocardial lesions did not reveal any features which could be considered as typical for Coxsackie B-3 infection. Further, the histological preparations had been scrutinized for qualitative differences in the myocarditis according to the age at inoculation. Although it was possible to distinguish three types of myocardial lesions, none of them was confined to any age group.

Type 1 lesion.—The heart muscle fibers appeared to be undergoing hyalin degeneration and necrosis. Many nuclei showed pycnosis and karryorhexis. Among the fragmentary muscle fibers and debris one could see quite a few mononuclears (Figs. 3 and 4).

Type 2 lesion.—The damage was observed around a blood vessel, and quite often, it was combined with periarteritis (Figs. 5 and 6).

Type 3 lesion.—Most of the necrotic material had been replaced by connective tissue with a few mononuclears here and there (Figs. 7 and 8).

Type 1 myocardial lesions predominated and were observed in all age groups inoculated with Coxsackie B-3 even in those 63- to 70-day-old mice which had been inoculated with normal passaged mouse carcass suspension. The lesions in this latter group, however, belonged mostly to type 2. Type 3 lesions were quite rare and were distributed at random.

Discussion

The outstanding feature of the results recorded is the finding of myocarditis, observed microscopically, and often macroscopically, in 100% of inoculated mice in the age groups from 12 days onwards to the 56th day of life. This complete susceptibility stands in marked contrast to the incidence of myocarditis recorded in the literature for any age group (6, 10, 11, 22) and was achieved by Kilbourne in 35-day-old mice only with the aid of cortisone (21).

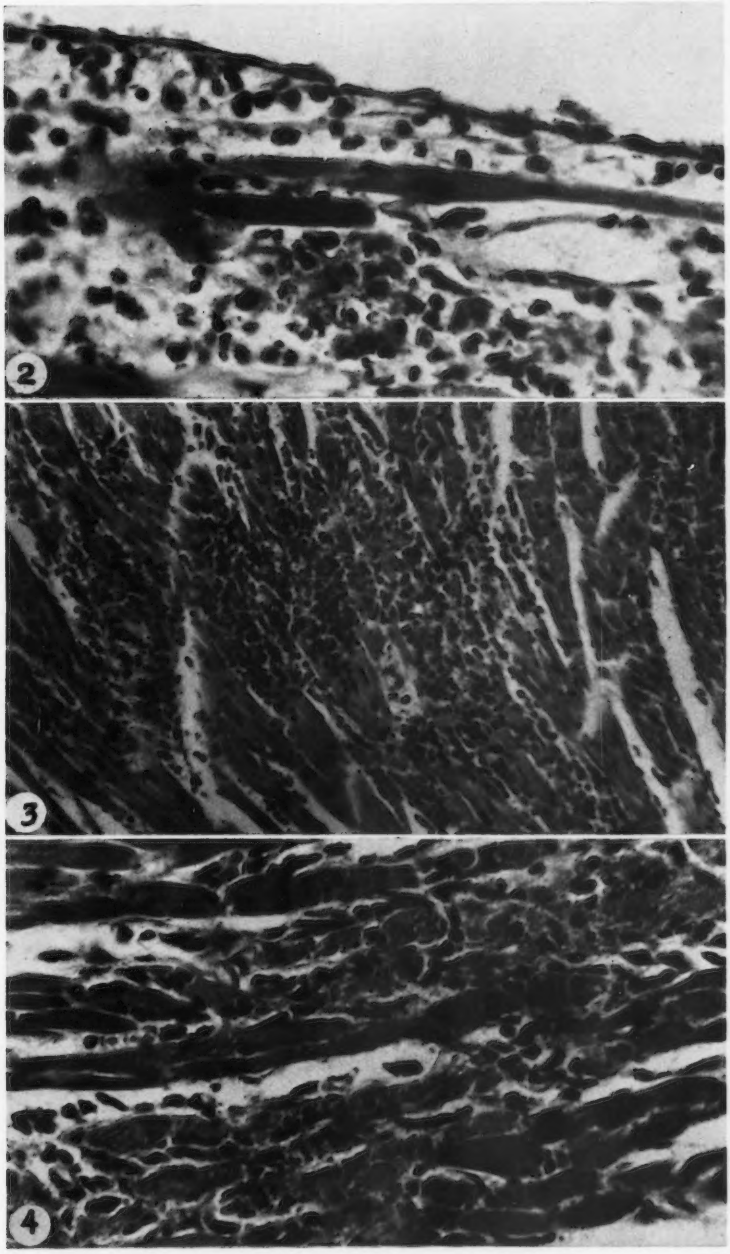

FIG. 2. Pericarditis, H.E. 300×
FIG. 3. Type 1 myocardial lesion in experimental Coxsackie B-3 infection, H.E. 125×
FIG. 4. Magnification of Fig. 3. 300×

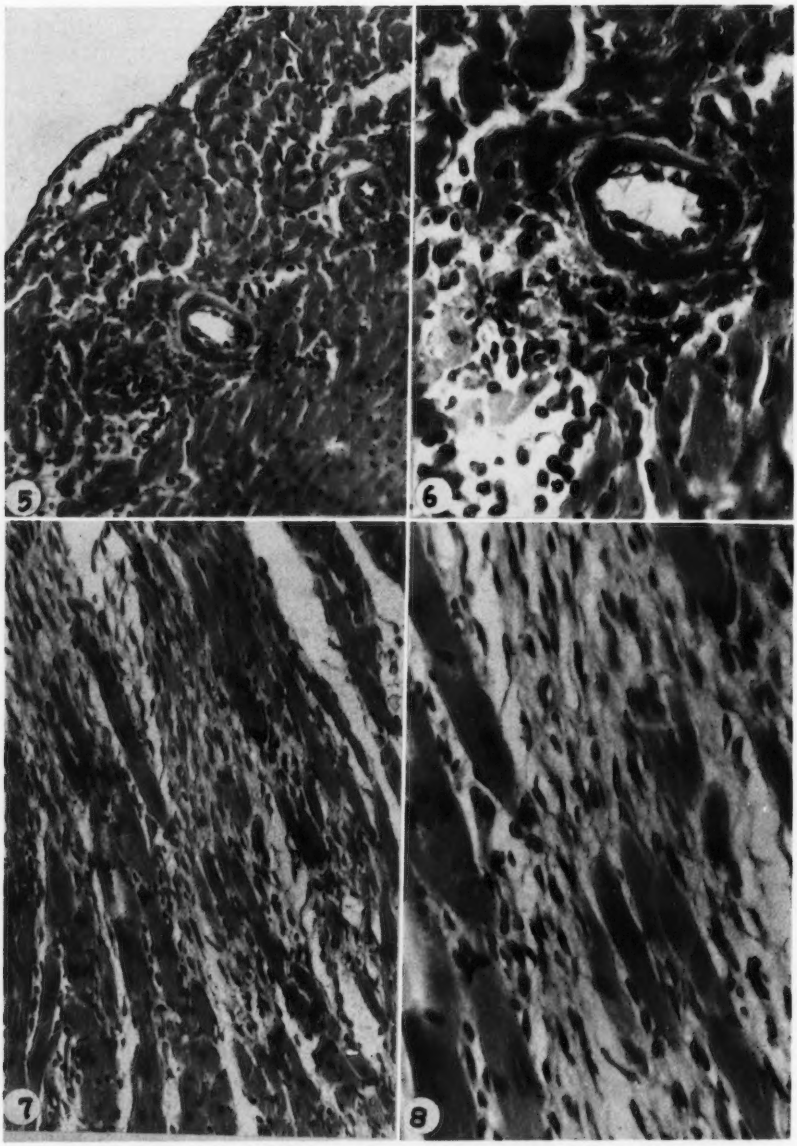

FIG. 5. Type 2 myocardial lesion in experimental Coxsackie B-3 infection, H.E. 125×
FIG. 6. Magnification of Fig. 5. 300×
FIG. 7. Type 3 myocardial lesion in experimental Coxsackie B-3 infection, H.E. 125×
FIG. 8. Magnification of Fig. 7. 300×

Equally striking is the absence of myocardial lesions during the first days of life when the infected hearts were examined not later than 7 days after inoculation.

Differences of this degree could not be accepted without profound testing and evaluation of the various factors involved in the experimental Coxsackie B-3 infection. The additional tests have clearly shown that the high incidence of myocarditis in weaned and adult mice is a permanent manifestation of the Coxsackie B-3 virus infection under investigation, irrespective of the route of inoculation, inoculating medium, virus concentration, and sex of the host. Seven days appear to be a sufficient length of time for a complete development of the Coxsackie B-3 myocarditis. The severity of the lesions in mice examined 2 weeks after inoculation and later is reduced, most likely due to repair.

The offspring of the albino and wild mouse when inoculated with Coxsackie B-3 show as high a frequency and severity of myocarditis as the control-stock mice. Yet mice from the Albany colony appear to be more resistant and, if changes in the myocardium are found, they are quite minute. From the information so far available, one would be justified to conclude that the high susceptibility of myocardium to Coxsackie B-3 virus is due to an intrinsic factor(s) in the heart tissue of our stock mice which have not been eliminated by crossing with a wild mouse.

Another factor, of course, can be a special affinity of the particular Coxsackie B-3 strain, which has been used in all these investigations, for the heart muscle. This aspect could be clarified by using other strains and types of Coxsackie group viruses and work in this respect is in progress.

Whether the related findings are due to the particular susceptibility of our mice or whether the Coxsackie B-3 is a cardiotropic mutant, the fact remains that we are working with a host–virus model, which enables us to imitate the Coxsackie–human relationship (3, 7–9, 13–15, 17, 18, 24–26, 28–33, 35). Furthermore, these findings allow of an experimental approach towards the elucidation of the intrinsic factors which influence the susceptibility of the myocardium.

In this respect it seems appropriate to call to mind the rather peculiar response of the myocardium to the inoculation of normal *passaged* mouse carcass suspension. This inoculation produces myocarditis with periarteritis in 5 out of 19 inoculated adult mice 7 days after inoculation Yet the same inoculum does not cause any changes in mice up to 15-day-old animals which are highly susceptible to Coxsackie B-3 in tissue culture fluid. One might postulate the presence of a latent infective agent in the tissues but as yet such an agent has not been isolated or identified, and the random occurrence of myocarditis had not been observed in normal uninoculated mice.

The observed morphological character of the mouse myocardial lesions corresponds to the description of post-mortem findings in the Coxsackie myocarditis in newborn children (3, 7, 13, 14, 18, 23), a fact which encourages the further study of this host–virus relationship.

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MOLECULAR HYDROGEN AND NITROGEN FIXATION BY CLOSTRIDIUM PASTEURIANUM[1]

D. W. S. WESTLAKE[2] AND P. W. WILSON

Abstract

The total amount of nitrogen fixed and the efficiency of nitrogen fixation by strain *Clostridium pasteurianum* W5 have been increased by using improved cultural conditions. Also, consistent growth of the organism in shake culture has been obtained. Evidence is presented supporting the view that hydrogen is a specific, competitive inhibitor of nitrogen fixation in this organism. The K_m for nitrogen fixation is around 0.03 atmosphere and the estimated K_i for hydrogen inhibition is 0.5 ± 0.05 atmosphere, a value considerably higher than that found in the other nitrogen-fixing systems studied.

Introduction

Molecular hydrogen is a unique specific, competitive inhibitor of the nitrogen-fixing systems in different agents (see review in ref. 10). However, the early experiments with the active hydrogen-producing organisms *Clostridium pasteurianum* and *Rhodospirillum rubrum* indicated that molecular hydrogen did not markedly affect the nitrogen fixation process in those two species. For example, Rosenblum and Wilson (7) reported that 60% hydrogen decreased the efficiency and the total amount of nitrogen fixed by *C. pasteurianum* but did not affect the rate of fixation. Shug, Wilson, and Hamilton (8), however, obtained spectrophotometric evidence that relatively high partial pressures of hydrogen (i.e., 0.8 atmosphere) blocked the initial steps of nitrogen fixation. That molecular hydrogen does inhibit the nitrogen-fixing system in *Clostridium* is evident from the experiments reported in this paper. Moreover, Pratt and Frenkel (6) have recently established hydrogen inhibition of nitrogen fixation by *R. rubrum*.

Method and Materials

The technique devised by Pengra and Wilson (5) to obtain consistent and reproducible growth of *A. aerogenes* in shake culture was used for the cultivation of *C. pasteurianum*. To minimize the effect of metabolically produced hydrogen, which could affect the determination of the velocity and the dissociation constants, short-term experiments were run. An 8-liter culture of *C. pasteurianum* was grown into the early logarithmic phase on a synthetic medium (sucrose, 20 g/l.; K_2HPO_4, 15.66 g/l.; KH_2PO_4, 1.36 g/l.; $MgSO_4.7H_2O$, 0.25 g/l.; $CaCl_2$, 0.05 g/l.; Fe ($FeSO_4.5H_2O$), 3 p.p.m.; Mo ($NaMoO_3$), 1.5 p.p.m.; and 2 μg biotin/l.). Fifty milliliters of this growing culture were transferred to 500-ml Erlenmeyer flasks equipped with sidearms and stoppers;

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after having the desired mixtures of H_2 and N_2 placed in them, these were incubated for 4 hours at 30° C on a Brunswick rotary shaker. The initial and final nitrogen content of the medium was determined by the Kjeldahl technique described by Wilson and Knight (12) and the rate of fixation determined from the formula:

$$v = \frac{2.303}{t \text{ (hours)}} \log_{10} \frac{\text{N final}}{\text{N initial}}.$$

The straight line obtained from the double reciprocal plot of the data ($1/v$ versus $1/pN_2$) was determined by the method of least squares.

Coincident with these growth experiments, a study was made of the effect of molecular hydrogen on the uptake of N^{15}-labelled nitrogen gas. Six-milliliter samples from a culture in the exponential phase of growth were exposed in duplicate with shaking for 30 minutes at 30° C, to 0.1 atmosphere of N_2^{15} (32 atom per cent excess) and various partial pressures of hydrogen. The duplicate samples were analyzed essentially by the methods described by Burris and Wilson (2). The precision of the instrument (Consolidated-Nier isotope ratio mass spectrometer) is about 0.005 atom per cent; an increase of 0.015 atom per cent excess was regarded as significant fixation.

Results and Discussion

Under the described cultural conditions *Clostridium pasteurianum* would fix up to 200 μg of nitrogen per milliliter of medium in 24 hours with an efficiency of 11 to 12 μg of nitrogen fixed per milligram of carbohydrate decomposed.

The results given in Table I clearly established an inhibition of nitrogen fixation by *C. pasteurianum* when relatively high partial pressures of H_2 are used. A few trials were made to determine if low partial pressures (less than 0.1 atmosphere) might be stimulatory, but as is evident from the data, no such effect was detected.

TABLE I

Effect of molecular hydrogen on fixation of N_2^{15} by whole cells of *C. pasteurianum**

		Atom % N^{15} excess				
pN_2	pH_2	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
0.1	0.0	2.139	0.437; 0.325	0.217; 0.265	0.592; 0.535	0.666; 0.620
0.1	0.01	—	—	0.272; 0.253	—	—
0.1	0.03	—	—	—	—	0.623; 0.664
0.1	0.05	—	—	—	0.560	—
0.1	0.10	—	0.419; 0.402	0.253	—	0.548
0.1	0.3	—	0.401	—	—	—
0.1	0.5	—	0.389	—	0.515; 0.454	0.486; 0.474
0.1	0.6	1.857	—	—	—	—
0.1	0.8	—	—	0.161; 0.197	—	—
0.1	0.9	1.457	0.171	—	—	—

*Cells exposed at 30° C for 30 minutes. Difference between pN_2, pH_2, and atmospheric pressure made up with helium.

Figure 1 indicates that molecular hydrogen is a *competitive* inhibitor of nitrogen fixation in *C. pasteurianum*. Although the three lines do not have the same ordinate intercept, as is required for this type of inhibition, this discrepancy probably arises from technical difficulties involved in preparing the gas mixtures and from errors in determining small increases in total nitrogen. Other experiments were performed comparing 85% and 0% hydrogen which did show the same intercept. The data in Fig. 2 confirm the previous results of Rosenblum and Wilson (7) and Zelitch (14) that molecular hydrogen has no effect on the rate of ammonia utilization by this organism. Therefore, as in the other nitrogen-fixing systems where this inhibitor has been tested, it is specific for the nitrogen-fixing process.

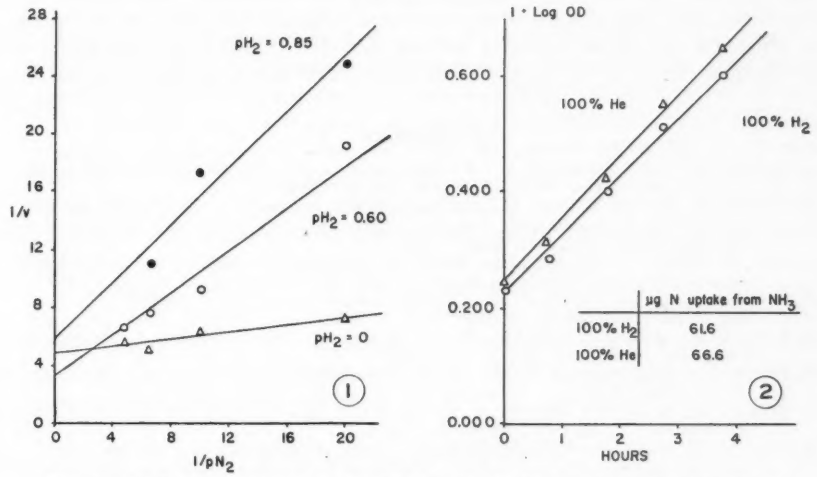

FIG. 1. Burk-Lineweaver plot of inhibition by hydrogen of nitrogen fixation in *Clostridium pasteurianum*.

FIG. 2. Effect of molecular hydrogen on the growth of *Clostridium pasteurianum* with ammonia as the source of nitrogen.

The estimation of the K_m for nitrogen fixation by this organism using the formula, slope $= K_m/V$, and the data in Fig. 1 yields 0.03 atmosphere, a value in good agreement with that reported for anaerobic nitrogen fixation by a strain of *Bacillus polymyxa* (3) as well as being of the same order as that reported for aerobic fixation by *Azotobacter vinelandii* (11), by the red clover symbiotic system (9), and by *Nostoc muscorum* (1).

Although the data in Fig. 1 show an effect by the presence of 60% H_2, those in Table I indicate that only occasionally would a pH_2 of 60% yield inhibition which would be detectable in growth studies. This observation affords a basis for the estimation of the K_i for hydrogen inhibition in this organism. Assuming that an inhibition of 20% would be detectable with a pH_2 of 0.6 atmosphere and a pN_2 of 0.1 atmosphere the K_i can be calculated from the formula (4):

$$\frac{v}{v_i} = 1 + \frac{K_m}{K_i}\frac{I}{K_m + S},$$

$$\frac{1.00}{0.80} = 1 + \frac{0.03}{K_i}\frac{0.6}{0.03 + 0.1},$$

$K_i = 0.5$ atmosphere with an estimated error of about 10%.

This estimated K_i for hydrogen inhibition of nitrogen fixation by *C. pasteurianum* is much higher than the 0.11 atmosphere reported for *A. vinelandii* (13), the 0.15 atmosphere reported for the red clover symbiotic system (9), and the 0.1 atmosphere reported for *B. polymyxa* (3). This observation suggests that the organism has developed a compensatory mechanism for minimizing the effect of hydrogen gas on the nitrogen fixation process, therefore avoiding the metabolic error of having a major product of carbohydrate metabolism affecting the process of nitrogen assimilation by the organism.

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MICROBIAL DECOMPOSITION OF RUTIN[1]

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Abstract

A number of molds, streptomycetes, and bacteria, obtained from culture collections and by enrichment techniques, were tested for their ability to degrade rutin. The molds, particularly *Aspergillus flavus* and *A. niger*, appeared to be more active than either the streptomycetes or bacteria. The aspergilli when grown on either rutin or quercetin produced extracellular enzymes that degraded both rutin and quercetin but not quercitrin. Rutinose, protocatechuic acid, phloroglucinol carboxylic acid, and a phloroglucinol carboxylic acid – protocatechuic acid ester were identified by paper chromatography as the products.

Introduction

Although the chemistry of flavones and related compounds has been the subject of extensive chemical investigations, relatively little information is available on the mechanism of biological degradation of these compounds. In vitro and in vivo experiments dealing with the degradation of rutin and quercetin in animals have shown that these compounds are readily metabolized to water-soluble products (3, 4, 11, 12). Huang (9, 10) and Harborne and Sherratt (8) described the activity of a fungal "anthocyanase" found in the culture medium of *Aspergillus* species that rapidly decolorized anthocyanins producing anthocyanidins and sugars.

This paper describes the results of a survey conducted to determine what organisms are capable of attacking rutin and of a study of three molds that produce extracellular enzymes capable of degrading rutin to rutinose, phloroglucinol carboxylic acid, protocatechuic acid, and a phloroglucinol carboxylic acid – protocatechuic acid ester.

Materials and Methods

Bacteriological

One hundred isolates capable of degrading rutin were obtained by enrichment techniques. The enrichment procedures consisted of: (*a*) inoculating broth containing 0.15% rutin with spruce litter, soil from a field of buckwheat or from an oil refinery, then when good growth was observed, serially transferring the cultures three or four times and finally plating on agar containing 1% rutin; or (*b*) adding rutin directly to moist soil, incubating the mixture at room temperature for a few weeks, and then plating on rutin agar. The first procedure resulted in the isolation of a number of bacteria, whereas the second procedure favored the isolation of molds and streptomycetes. Those colonies surrounded by clear zones were picked and replated on rutin agar, then transferred to rutin broth and rutin slants. Agar plates exposed to air yielded molds and streptomycetes but few bacteria. In addition to

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these isolates, a number of cultures were selected from the Prairie Regional Laboratory culture collection and tested for their ability to decompose rutin.

All media contained inorganic salts (100 mg $MgSO_4.7H_2O$, 1.0 mg each of $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$, 0.75 mg $MnSO_4.H_2O$, and 0.05 mg $CuSO_4.5H_2O$ per liter) and vitamins (500 μg each of thiamine hydrochloride, riboflavin, calcium pantothenate, and niacin; 100 μg each of pyridoxal, para-aminobenzoic acid, and inositol; 10 μg of folic acid; and 4 μg of biotin per liter). The basal medium used for enrichments contained 0.15% K_2HPO_4, 0.05% $NH_4H_2PO_4$, 0.01% N-Z-amine, salts, and vitamins. Stock cultures were maintained on agar slants containing in addition to the above 0.05% yeast extract and 0.015% rutin. A broth of similar composition was used to prepare the inoculum.

The medium employed for determining the utilization of rutin and the production of the extracellular enzymes consisted of 0.2% rutin, 0.15% $(NH_4)_2HPO_4$, 0.05% KH_2PO_4, 0.1% N-Z-amine, vitamins, and salts. The rutin was weighed directly into each 250-ml Erlenmeyer flask and sterilized separately with 20 ml of water as rutin is decomposed by autoclaving in the presence of salts. The remaining ingredients were prepared at five times the final concentration, sterilized, and 5 ml added aseptically to each flask prior to inoculation.

The decomposition of rutin by growing cultures was determined by inoculating 25 ml of the test medium with 0.5 ml of a 48-hour-old culture and incubating the flasks for 72 hours at 30° C on a Gump rotary shaker (110 r.p.m., $1\frac{1}{4}$ in. eccentricity). The cultures were then diluted to 100 ml with ethanol, shaken until the undecomposed rutin was dissolved, and then suitable aliquots taken for rutin analysis.

Enzymatic

Culture filtrates, used for determination of extracellular enzymes, were obtained by passing 72-hour-old cultures through Whatman No. 2 filter paper and then through either an ultrafine fritted glass funnel or through a Millipore filter (0.45 mμ pore size). Seitz filter pads were not satisfactory as they absorbed the extracellular enzymes. When necessary, these filtrates were stored at −17° C. Extracts of bacteria were obtained by treating at 5° C cell suspensions in a 10-kc Raytheon oscillator.

Glycosidase activity was measured by mixing 1 ml of culture filtrate with 4 mg of rutin suspended in 1 ml of buffer and determining the reducing activity after incubation for 1 hour at 30° C. Phosphate buffer (0.1 *M*, pH 6.5) was used for the bacterial filtrates, and acetate buffer (0.1 *M*, pH 5.5) for mold filtrates. The mixture was shaken during incubation to maintain the rutin in suspension and the reaction was stopped by the addition of 0.2 ml of *N* NaOH. Controls contained enzyme inactivated by heating in a boiling water bath for 5 minutes. The reducing sugar was determined by the ferricyanide reduction method using a Technicon autoanalyzer.

The ability of culture filtrates and cell extracts to degrade rutin, quercetin, or quercitrin was determined by incubating 2 ml of the filtrate with 20 mg

of the flavonol with shaking for 3 hours at 30° C. The reaction mixture was then diluted with ethanol to 100 ml and a suitable aliquot taken for determination of the remaining flavonol.

Analytical

The flavonols were determined by the $AlCl_3$ method (7). One milliliter of the alcohol-solubilized sample, suitably diluted to contain not more than 100 μg of rutin or quercitrin or 50 μg of quercetin per milliliter, was mixed with 5 ml of 0.02 *M* $AlCl_3$ and allowed to stand for 30 minutes at room temperature. The optical density was measured at 415, 405, and 440 mμ for rutin, quercitrin, and quercetin, respectively.

Chromatographic

The products obtained from the degradation of rutin could be separated by chromatography on Whatman No. 4 paper using acetic acid – water (15:85). The compounds were located by exposure to ammonia and ultraviolet light and by treatment with diazotized sulphanilic acid, heat, and sodium carbonate (2). Rutinose was identified by paper chromatography using an ethyl acetate – acetic acid – water solvent (9:2:2) and a silver nitrate spray.

Rutinose was prepared by hydrolysis of rutin with acetic acid (16) and by enzymatic hydrolysis with culture filtrates of *Aspergillus flavus* PRL 1720. After hydrolysis, the aromatic residues were extracted with ether, the sugar absorbed on a coconut charcoal column and gradiently eluted with 5 to 10% ethanol, then concentrated to a thick sirup (1). Protocatechuic acid was prepared from vanillin and purified by sublimation (13). The phloroglucinol carboxylic acid – protocatechuic acid ester was isolated by precipitation at pH 1.5 from an enzymatic hydrolyzate of rutin by culture filtrates of *A. flavus* PRL 1720.

Experimental

Survey for Microorganisms Capable of Degrading Rutin

Two hundred and eighty cultures, some obtained from the Prairie Regional Laboratory culture collection, some by enrichment techniques, and others by exposing rutin-containing agar plates outdoors, were tested for their ability to decompose rutin. The following genera, with the number of cultures tested in parentheses, did not attack rutin: *Absidia* (1), *Armillaria* (2), *Ascochyta* (1), *Botryotrichum* (1), *Chaetomium* (1), *Claviceps* (1), *Coccospora* (1), *Darluca* (1), *Dibotryon* (1), *Fomes* (1), *Lentinus* (1), *Leptosphaeria* (1), *Mucor* (2), *Phycomyces* (1), *Rhizoctonia* (1), *Rhizopus* (2), *Stachybotrys* (1), *Trametes* (1), *Trichoderma* (1), *Trichothecium* (1), *Ustilago* (1), *Verticillium* (2), *Hansenula* (2), *Saccharomyces* (8), *Torulopsis* (2), *Aerobacter* (4), *Agrobacterium* (3), *Alcaligenes* (1), *Erwinia* (4), *Flavobacterium* (4), *Leuconostoc* (2), *Micrococcus* (4), and *Vibrio* (1).

The genera that did attack rutin are listed in Table I. Most of the bacteria obtained by enrichment techniques were Gram-negative rods although a few bacilli and micrococci were isolated. Three motile Gram-negative rods

TABLE I

Ability of microorganisms to degrade rutin

Genera	Number of cultures			
	Tested	Negative	Utilizing <50% of rutin	Utilizing >50% of rutin
Alternaria	1	0	0	1
Aspergillus	14	0	3	10
Botrytis	1	0	1	0
Calonectria	1	0	1	0
Cephalosporium	2	1	0	1
Diaporthe	1	0	1	0
Dicoccum	1	0	1	0
Fusarium	5	2	2	1
Macrophomina	1	0	1	0
Penicillium	4	2	1	1
Polyporus	1	0	1	0
Pullularia	1	0	0	1
Stemphylium	1	0	1	0
Thielaviopsis	1	0	1	0
Streptomyces	51	27	24	0
Bacillus	35	33	2	0
Corynebacterium	25	21	4	0
Pseudomonas	11	5	3	3
Xanthomonas	2	1	1	0
Unidentified molds	18	0	18	0
Unidentified bacteria	59	0	55	4

that completely degraded the rutin in the test medium were identified as *Pseudomonas* spp. and a fourth non-motile rod was tentatively identified as *Flavobacterium* sp. Although a large number of *Streptomyces* spp. obtained by enrichment from the culture collection utilized rutin, none decomposed more than half of the substrate supplied. The molds, however, were much more active, particularly cultures of the *A. flavus-oryzae* and *A. niger* groups.

Glycosidase Activity

The initial attack on rutin by microorganisms might be the hydrolysis of the glycosidic bond between the aglycone, quercetin, and the disaccharide, rutinose. Those cultures that were capable of degrading more than 50% of the rutin in the initial survey were examined for the production of extracellular glycosidases. The results of this survey are presented in Table II.

In general, few filtrates from mold cultures or extracts of bacterial cells contained active glycosidases or showed evidence of significantly attacking rutin. Three molds, however, did produce extracellular enzymes that released rutinose and also degraded the insoluble rutin to water-soluble products (Table II). Two of these molds, *A. flavus* (PRL 72) and *A. niger* (PRL 18), were obtained from the culture collection, while *A. flavus* (PRL 1720) was isolated from rutin enrichment of spruce litter.

When the three molds were grown on glucose, rhamnose, or rutinose, the culture filtrates did not hydrolyze α-methylglucoside or maltose, but very slowly attacked salicin, cellobiose, and rutin. This suggests the presence of a small amount of β-glycosidase. When the molds were grown on rutin,

TABLE II

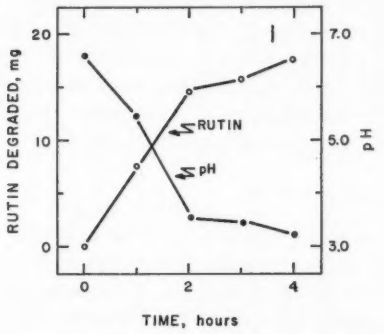
Glycosidic activity of cultures capable of utilizing rutin

	Rutin degraded, %	Filtrate, glycosidic activity	Filtrate, solubilizing activity
Alternaria sp. PRL 99	41	Trace	0
Aspergillus flavus PRL 72	98	+++	+++
Aspergillus flavus PRL 185	99	Trace	Trace
Aspergillus flavus PRL 1720	99	+++	+++
Aspergillus niger PRL 18	99	+++	+++
Aspergillus niger PRL Z3	77	+	Trace
Aspergillus niger PRL Z15	78	0	Trace
Aspergillus niger PRL Z16	89	+	Trace
Cephalosporium sp. (j)	98	0	0
Diaporthe sp. PRL 1447	89	Trace	Trace
Fusarium sp. PRL 19	92	Trace	Trace
Fusarium sp. (I)	97	Trace	Trace
Penicillium sp. PRL 188	60	Trace	Trace
Penicillium sp. (B)	96	0	0
Pullularia sp. (F)	98	0	+
Pseudomonas sp. (7)	96	Trace	0
Pseudomonas sp. (44)	96	Trace	0
Pseudomonas sp. (51)	96	Trace	0
Flavobacterium sp. (55)	95	Trace	Trace

quercetin, or quercitrin, however, the culture filtrates were 50 times more active, indicating that the β-glycosidase was an adaptive enzyme, apparently induced by the aglycone. Alpha-glycosidase activity was again absent.

Degradation of Rutin by Culture Filtrates

Filtrates from the three *Aspergillus* species grown on rutin were capable of degrading rutin at the rate of approximately 3 mg per hour per milliliter of culture filtrate. The decomposition of rutin was accompanied by a sharp drop in the pH of unbuffered reaction mixtures (Fig. 1). A similar drop in pH also occurred with the degradation of quercetin (Fig. 2).



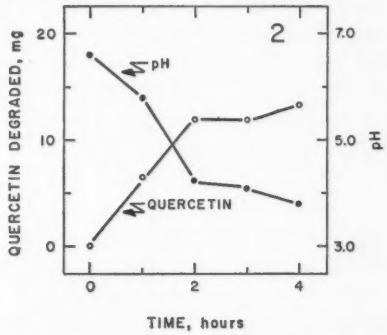


FIG. 1. Change in pH with rutin degradation. Reaction mixture: 2 ml of culture filtrate from PRL 1720 and 20 mg rutin. Similar results obtained with filtrates from PRL 18 and PRL 72.

FIG. 2. Change in pH with quercetin degradation. Reaction mixture: 2 ml of culture filtrate from PRL 1720 and 20 mg quercetin. Similar results were obtained with filtrates from PRL 18 and PRL 72.

TABLE III

Chromatographic identification of products

Sample	Solvent	R_f	Treatment		
			$AgNO_3$ spray	NH_3-UV	Diazotized sulphanilic acid
Filtrate 1720	Ethyl acetate – acetic acid – water	0.07	Greyish black	—	—
Rutinose	Ethyl acetate – acetic acid – water	0.07	Greyish black	—	—
Filtrate 1720	Acetic acid – water	0.57	—	Bluish-white fluorescence	Dark-brown spot
Phloroglucinol carboxylic acid	Acetic acid – water	0.57	—	Bluish-white fluorescence	Dark-brown spot
Filtrate 1720	Acetic acid – water	0.51	—	Deep-blue fluorescence	Brown spot
Protocatechuic acid	Acetic acid – water	0.51	—	Deep-blue fluorescence	Brown spot
Filtrate 1720	Acetic acid – water	0.45	—	Pale white	Light-tan spot
Phloroglucinol carboxylic acid – protocatechuic acid ester	Acetic acid – water	0.45	—	Pale white	Light-tan spot

Filtrates grown on either rutin or quercetin were capable of degrading both rutin and quercetin, but not quercitrin (3-rhamnoside of quercetin). Filtrates from cultures grown on glucose, rhamnose, rutinose, protocatechuic acid, or phloroglucinol did not contain enzymes capable of attacking rutin, quercetin, or quercitrin. The extracellular enzyme system produced by these molds thus appears to be specifically induced by rutin or the aglycone, quercetin.

Oxygen was required by the extracellular enzyme systems for the degradation of rutin and quercetin. When the atmosphere above the reaction mixtures was replaced with nitrogen or a mixture of 5% carbon dioxide and nitrogen the decomposition of rutin and quercetin was inhibited (Fig. 3). However, neither the uptake of oxygen nor the production of carbon dioxide could be demonstrated by conventional manometric techniques although 10 mg of rutin was degraded.

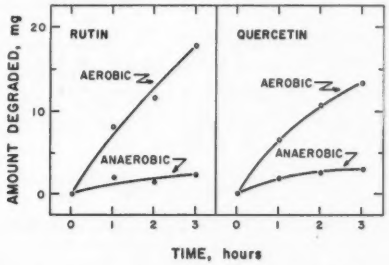


FIG. 3. Comparison of aerobic and anaerobic conditions on the degradation of rutin and quercetin. Reaction mixture: 2 ml of culture filtrate from PRL 1720 and 20 mg rutin or quercetin. Similar results were obtained with filtrates from PRL 18 and PRL 72.

Products of the Enzymatic Degradation of Rutin

The products from the degradation of rutin by the extracellular enzyme systems were identified by paper chromatography and are presented in Table III. Details of chemical studies that confirmed the identity of these products will be published in a subsequent paper. Quercetin was not detected in these reaction mixtures by either paper chromatographic or spectrophotometric techniques. The products of the enzymatic degradation of quercetin are the same as those obtained from rutin with the exception of rutinose.

Discussion

Molds appear to be more active than either streptomycetes or bacteria in degrading rutin. Not only were molds most numerous on plates of rutin agar exposed to air and to soil but also, of the organisms tested, only two molds, *Aspergillus flavus* and *A. niger*, produced a strong extracellular enzyme system capable of degrading rutin.

The initial attack on rutin by these extracellular enzyme systems apparently is the hydrolysis of the glycosidic bond by an adaptive β-glycosidase (Fig. 4). Production of this enzyme is stimulated by the aglycone rather than by the

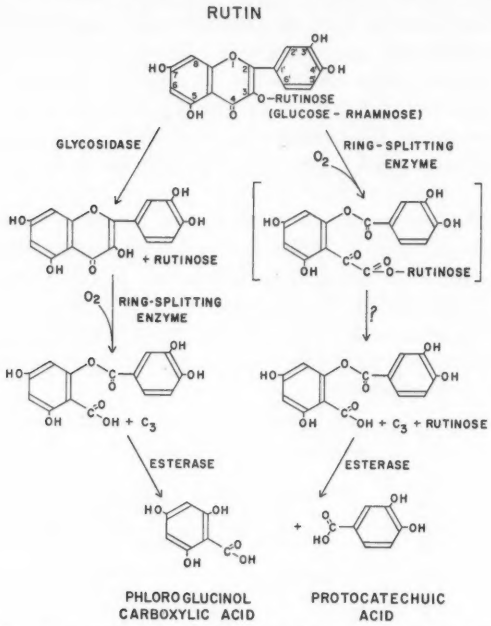


FIG. 4. Outline of possible routes for the degradation of rutin by microorganisms.

sugar. Rutinose is released, but is not degraded into its component sugars as would be expected if the bond between rhamnose and glucose was of the β-configuration as reported in the literature (14, 15). This bond is more likely α than β as the culture filtrates do not contain an α-glycosidase. This has been confirmed by independent chemical studies (6). The aglycone, quercetin, on the other hand, has not been detected in hydrolyzates of rutin. Relatively weak glycosidase activity followed by strong ring-opening activity would, however, prevent the accumulation of quercetin.

Alternatively, the initial attack on rutin may be cleavage of the heterocyclic ring between carbon-2 and -3 (Fig. 4). Rutinose would then be released from the unstable α-keto-ester resulting from the initial oxidative attack. This pathway also accounts for the failure to detect quercetin as an intermediate in the degradation of rutin.

The inability of filtrates from cultures grown on rutin or quercetin to attack quercitrin may be explained by assuming that the β-glycosidase is specific for glucosides and will not hydrolyze rhamnosides. As the glycosidase is induced by the aglycone rather than the sugars, this explanation is not favored. Rather the glycosidic bond in quercitrin is suspected of being of the α-configuration and since the filtrates do not contain an α-glycosidase the compound cannot be degraded. Alternatively, if the initial attack is opening of the ring between carbon-2 and -3, rhamnose may be so placed as to stearically prevent the ring-opening enzyme from acting. It is worth

noting that all three molds grew on and utilized quercitrin, but did not produce extracellular enzymes that would attack quercitrin, rutin, or quercetin.

The ester of protocatechuic acid and phloroglucinol carboxylic acid is the first stable intermediate identified in the degradation of rutin and quercetin. This ester is subsequently hydrolyzed by an esterase to phloroglucinol carboxylic acid and protocatechuic acid, compounds that are also produced by animal tissues acting on quercetin (3, 4). Pathways for the degradation of these compounds have been established (5).

Rutinose, phloroglucinol carboxylic acid, and protocatechuic acid account for 26 of the 27 carbons present in the rutin molecule. Attempts to locate the remaining carbon, carbon-3, as carbon dioxide, formic acid, formaldehyde, or methanol failed. Rutin specifically labelled with carbon-14 at position-3 is being prepared to aid in solving this problem.

Acknowledgments

We wish to express our grateful appreciation to Drs. R. H. Haskins and W. A. Taber, who provided the molds used in this study as well as identifying some we obtained by enrichment procedures. We also wish to thank the technicians, Miss C. Van Loenan and Mr. A. S. Sieben, for their assistance in this work.

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PROTEOLYTIC ACTIVITY OF BACILLUS SUBTILIS IN A CLAY–PROTEIN PASTE SYSTEM ANALOGOUS TO SOIL[1]

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Abstract

The proteolytic specificity of a purified proteinase, Nagarse, of *Bacillus subtilis* N′ towards denatured lysozyme was compared with that of the organism growing in a model soil system consisting of a heat-denatured lysozyme–kaolinite complex in a paste form which represents the natural habitat of soil organisms. The use of a sterile technique and a specially designed apparatus permitted periodic withdrawal of nonadsorbed and nonassimilated proteolysis products from the medium; such products were formed by the proteolytic activity of exoenzymes excreted by *B. subtilis* N′. A chromatographic comparison was made between the proteolysis products of *B. subtilis* N′ growing in the lysozyme–kaolinite system and those of the proteinase Nagarse acting on denatured lysozyme in solution; some differences were observed. The growth rate studies indicated that the lag period of growth of *B. subtilis* N′ in the lysozyme–kaolinite complex extended up to one day. The bacteria excreted exoenzymes during this lag period, which hydrolyzed the substrate protein, but the proteolysis products were not assimilated until the exponential growth period started.

Introduction

In the soil, bacteria live mainly on the surface of the soil particles. An investigation of microbial metabolic activities in colloidal systems is an essential adjunct to the classical studies in solution cultures for understanding the biochemical activities in the soil. As an example of the complications involved when biological reactions occur at surfaces, the "pH effect" might be mentioned. The pH at the surface of the kaolinite particle is one to two units lower than in the ambient liquid, due to the concentrations of positive charges within the electric double layer formed at the negatively charged surface. This leads to an apparent difference in pH optimum for enzymatic activity, since the measured pH is that of the ambient fluid, not the surface. Unless the shift of the pH optimum is taken into account in assaying for enzymes in adsorbing media such as soil, a low estimation of enzymatic activity may result (13, 14, 15).

Some work with synthetic soil media for microbiological investigation has been reported (1, 16), and Lochhead and Thexton (11) have noted the excellent survival of bacteria adhering to soil. Estermann and McLaren (3) have reviewed the effect of adsorbing media on bacterial activities. We have reported elsewhere (4)[2] that *Bacillus subtilis* and members of the genera

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Contribution from the Department of Soils and Plant Nutrition, University of California, Berkeley, California. This work was presented in part before the Western Society of Soil Science at the American Association for the Advancement of Science Meeting, Pacific Division, San Diego, June 15–19, 1959. Paper VII in the series "Adsorption and reactions of enzymes and proteins on clays."

[2]We wish to draw attention to an error in the legend to Figure 2, reference 4. The concluding phrase "except for the ½ and ⅓ monolayers on montmorillonite" was omitted from the title.

Pseudomonas and *Flavobacterium* act on protein substrates adsorbed to kaolinite and bentonite and suspended in mineral salt solution, with the liberation of ammonia as a by-product. *B. subtilis* itself adheres to kaolinite particles. These observations, and commercial availability of the proteolytic enzyme, Nagarse, produced from *Bacillus subtilis* N′, suggested the possibility of a comparison between proteolysis products removed from a kaolinite–protein paste medium inoculated with *B. subtilis* N′, and the products produced by Nagarse acting on the same protein in solution. This report describes the experimental approach to such a problem and the results obtained. A moist, aerated paste consisting of Celite and heat-denatured lysozyme adsorbed on kaolinite, inoculated with *B. subtilis* N′, was used as a model for the natural habitat of soil organisms.

Materials and Methods

Bacterial Culture

A pure culture of *Bacillus subtilis* N′ was obtained from Dr. K. Okunuki of Osaka University, Japan.

Nutrient Media

Lysozyme-agar was prepared by adding 10 ml warm nutrient agar to 1 ml of 2 mg/ml heat-denatured lysozyme (Armour) solution in a sterile Petri plate. The protein appeared as a finely divided white precipitate.

Nutrient agar and nutrient broth were used for culture maintenance and plate counts.

The inorganic nutrient salt solution consisted of:

K_2HPO_4	3.20 g
$MgSO_4$	0.20 g
$CaSO_4.2H_2O$	0.25 g
$FeSO_4.7H_2O$	0.02 g
Micronutrient stock solution[3]	1 ml
Distilled water to	1 liter.

The pH of the solution was 8.2.

Bacterial Culture Apparatus

An apparatus (Fig. 1) was designed which permitted sterile aerobic growth of bacteria and sterile sampling of products released from the growth medium at any time. Sterile techniques were used throughout. The media, bacterial suspensions, and other reagents were introduced into the apparatus through the funnel A and sedimented on the sintered glass filter C. The foot B under the funnel outlet tube served to distribute the suspended material evenly on the filter. Products were collected in container D by vacuum filtration and withdrawn into E. The sampling technique is described more fully below. The air flow was governed by the applied vacuum and the stopcock system. During operation the air pressure in the apparatus was 670 mm Hg, as measured with manometer J. Vacuum release was facilitated by the

[3]The composition of micronutrient stock solution in g/l. is as follows: $MnSO_4.H_2O$, 3.20; H_3BO_3, 2.86; $ZnSO_4.7H_2O$, 2.20; H_2MoO_4, 0.017; $CuSO_4.5H_2O$, 0.079.

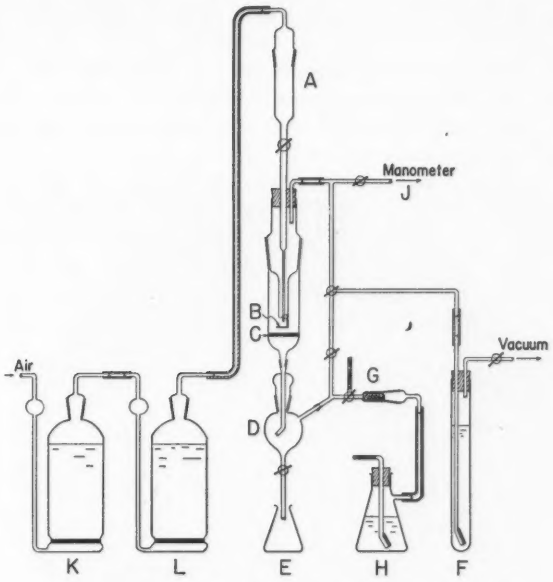

FIG. 1. The sterile, aerobic bacterial culture apparatus.

system G–H when desired. Entering air was sterilized when it passed through concentrated sulphuric acid (K) and then water vapor saturated in L. Ammonia released was collected at F. The apparatus was made of borosilicate glassware. The apparatus was sterilized as a unit by autoclaving for 30 minutes at 15 p.s.i.

Kaolinite–Lysozyme Growth Medium

Crystalline lysozyme (Armour) denatured by autoclaving 1 hour at 15 p.s.i., and Peerless electrodialyzed kaolinite were used as previously described (4). The sterile kaolinite – heat-denatured lysozyme – Celite (Johns-Manville Co.) suspension (abbreviated KHLC) was prepared as follows (4, 12): 6.5 ml water suspension, containing 0.094 g kaolinite and 0.2 g Celite, were sedimented, resuspended, and washed three times with 0.004 *M* phosphate buffer, pH 3.4. After the last centrifugation the pellet was resuspended in water and sterilized by autoclaving at 15 p.s.i. for 30 minutes. A 1.8-mg amount of heat-denatured lysozyme (taken from a sterile aqueous solution containing 2 mg/ml) was added to the sterile kaolinite–Celite suspension approximately ½ hour before inoculation with *Bacillus subtilis* N′. The adsorption of lysozyme to kaolinite was facilitated by slowly rotating the flask.

To prepare the bacterial inoculum for the bacterial culture apparatus, 10 ml of nutrient broth in an Erlenmeyer flask were incubated with added *B. subtilis* N′ inoculum for 48 hours on a shaker at room temperature. The 48-hour culture was washed three times with 10-ml quantities of sterile nutrient

salt solution followed by centrifugation. The washed cell suspension in nutrient salt solution was added to the sterile KHLC suspension and the bacterial cells were allowed to adsorb on the suspended solid particles for 10 minutes before the suspension was placed on the sintered glass filter (C, Fig. 1) in the apparatus. The pH of the paste as used in the apparatus was 7.5 as measured with a glass electrode.

At intervals, 5-ml portions of sterile inorganic nutrient salt solution were introduced through A, the direction of the air flow reversed (air inlet through system H–G–D), and the paste suspension was permitted to agitate in the air stream. After 15 minutes the air flow was reversed again (through system D–F), the filtrate was collected in D, and withdrawn into E. It was then acidified with hydrochloric acid to pH <2 to stop any further proteolytic activity.

Plate counts were used to determine the number of bacteria added to the KHLC medium in the bacterial culture apparatus. The bacterial inoculum contained 10^7 to 10^8 *B. subtilis* N′ cells. The number of bacterial cells removed at each sampling varied between 10^3 and 10^5.

Any contamination of the paste at the end of an experimental period was checked by plating a loopful of the KHLC medium suspended in sterile water on nutrient agar.

Crude Bacterial Proteinase Preparation

For the culture medium these three solutions were autoclaved separately:

Solution I. Salt solution prepared according to Güntelberg (6).

Solution II. Heat-denatured lysozyme solution, 5 mg lysozyme/ml.

Solution III. Glucose solution, 50 g glucose/100 ml, to which 0.05 ml of 85% phosphoric acid was added to avoid browning during sterilization.

Sterile solutions of I and II, 10 ml each, were mixed with 2 ml of sterile solution III in a sterile flask. The flask was inoculated with 1 ml of *Bacillus subtilis* N′ washed cell suspension in 0.85% saline solution and incubated on a shaker at room temperature. After 48 hours' incubation, the flask contents were centrifuged and the supernatant solution filtered through a Millipore filter (Millipore Filter Corp.). The filtrate was used for proteolytic degradation of lysozyme by incubating 10 ml of sterile heat-denatured lysozyme solution, 2 mg/ml, with 1 ml of sterile culture filtrate for 18 hours at room temperature.

Bacterial Proteinase, Nagarse

The crystalline bacterial proteinase, Nagarse, prepared from *Bacillus subtilis* N′, was obtained from Nagase and Co., Ltd., Amagasaki Factory, Amagasaki, Japan. The proteinase has been described by Hagihara, Matsubara, Okunuki, and co-workers (7, 8, 9, 17, 19). One milliliter of Nagarse solution containing 10 μg enzyme was added to 5 ml of heat-denatured aqueous lysozyme solution, pH 5.3, containing 2 mg lysozyme/ml, and incubated at room temperature for 18 hours. At the end of the incubation period the solution was tested for bacterial contamination and hydrochloric acid was added to stop further activity.

PLATE I

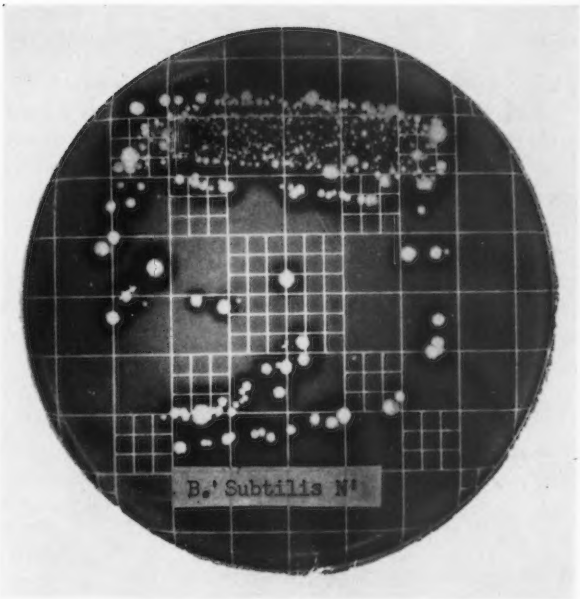

FIG. 2. Production of proteolytic exoenzymes by *Bacillus subtilis* N'.

Adsorption of Lysozyme Proteolysis Products on Kaolinite

Heat-denatured lysozyme solution, containing 2 mg protein initially in 2 ml solution and digested 24 hours by Nagarse, was added to 10 ml of washed kaolinite–Celite suspension containing 0.56 g kaolinite and 0.20 g Celite, and buffered at pH 8 with 0.01 *M* phosphate. The suspension was gently rotated for 1 hour and then centrifuged; the supernatant solution was acidified with hydrochloric acid to pH <2 and analyzed with a Beckman DU spectrophotometer for nonadsorbed protein.

Paper Chromatography and Electrophoresis of Lysozyme Proteolysis Products

Acidified samples of proteolysis products intended for paper chromatography were desalted according to the method of Haugaard and Haugaard (10). The proteolysis products were eluted from the ion exchange resin (Dowex 50-X8) column with 0.05 *M* ammonium hydroxide and a 5-ml aliquot of eluant of pH >6.5 was collected and evaporated at 90° C. The residue was dissolved in 0.05 ml of 0.01 *N* hydrochloric acid.

One-dimensional paper chromatography of the proteolysis products was performed by the descending method using *n*-butanol – acetic acid – water (4:1:5) mixture (20) as the developing solvent on Whatman No. 1 paper. Ninhydrin reagent by Moore and Stein (18) was used for the color development of spots.

Barbital buffer, ionic strength 0.05, pH 8.6, prepared according to Cremer and Tiselius (2), and 0.1 *M* phosphate buffer, pH 5.1, was used for open strip paper electrophoresis on Whatman 3MM paper in a Research Specialities Co. model 1400 apparatus at 500 v.

Ammonia Analysis

The amount of ammonia collected in the ammonia trap (F, Fig. 1) was determined by direct nesslerization.

Optical Density Measurements

The concentrations of proteins and proteolysis products in solutions were determined by optical density measurements at 2800 Å with a Beckman DU spectrophotometer. An extinction coefficient for lysozyme (5), $E^{1\%}_{2800}$, of 26.4 was used for lysozyme and its proteolysis products.

Results and Discussion

The excretion of proteolytic exoenzymes by *Bacillus subtilis* N′ was demonstrated by plating the organisms on a lysozyme agar medium (Fig. 2). Clear zones indicating digestion of protein appeared around the colonies and spread at a rate of about 3 mm per day until the entire plate was clarified.

Heat-denatured lysozyme was the only carbon and nitrogen source for *B. subtilis* N′ growing on the KHLC paste in the bacterial culture apparatus. As a consequence ammonia was produced by the organism (4). After 4 days about one-third of the total possible lysozyme proteolysis products (based on optical density measurements at 2800 Å) appeared in the filtrate (Table I) and after 6 days 15% of the total nitrogen appeared as free ammonia.

TABLE I

Elution of lysozyme proteolysis products from the kaolinite – heat-denatured lysozyme – Celite paste

Time of successive sampling, hours after inoculation	Protein products eluted,* mg
18	0.231
48	0.346
72	0.038
96	0.004

*Total lysozyme added: 1.80 mg.

The time sequence of chromatograms for *B. subtilis* N′ attack on KHLC (Fig. 3, rows III–VII) shows that the largest amount of products appears on the first day. This time sequence was also observed in analyzing filtrates for eluted proteolysis products by optical density measurements at 2800 Å (Table I).

In separate experiments using small inocula (about 10^5 organisms) bacterial numbers in the paste were found by plate counts to increase fourfold the first day and a hundredfold the second day. No contaminant organisms were observed. The plate counts suggest that the lag period of growth in this paste system extends up to one day. The results indicate that on the first day the organisms excrete proteolytic exoenzymes which act on lysozyme, but that the proteolysis products are not assimilated by the organisms until the exponential growth phase begins.

These experiments demonstrate that *Bacillus subtilis* N′ organisms can multiply in a soil-like paste, producing ammonia and protein degradation products from adsorbed protein as the result of exoenzyme activity. It was of further interest to observe whether the proteolytic products produced in this model soil system were comparable with those produced by the purified proteolytic enzyme (Nagarse) obtained from the same organism.

The commercially available *B. subtilis* N′ proteinase, Nagarse, was found to be partially adsorbed on KHLC at pH 8. The enzyme has been reported to be similar to chymotrypsin in proteolytic activity per unit weight toward denatured lysozyme (21). The broad pH optimum for Nagarse acting on casein in solution is pH 7–10 (17); on adsorbed, denatured lysozyme (KHLC), the optimum is at pH 8.5–9.5, at pH 6 50% of the maximum rate toward KHLC is observed (21). Although in our soil-like paste system the clay surface pH's may have been as much as 2 pH units lower than that of the buffer (13, 14, 15), the bacterial enzyme retained activity.

In Fig. 3 a time sequence of chromatograms obtained from the filtrates of *B. subtilis* N′ cultured in the apparatus on KHLC paste (rows III–VII) is compared with chromatograms of proteolysis products obtained from crystalline Nagarse acting on heat-denatured lysozyme in solution (rows I and II). Nagarse acting on heat-denatured lysozyme produced 15 peptide fractions as measured by one-dimensional chromatography. Using paper electrophoresis in pH 8.6 barbital, 14 fractions were observed. Spots numbered

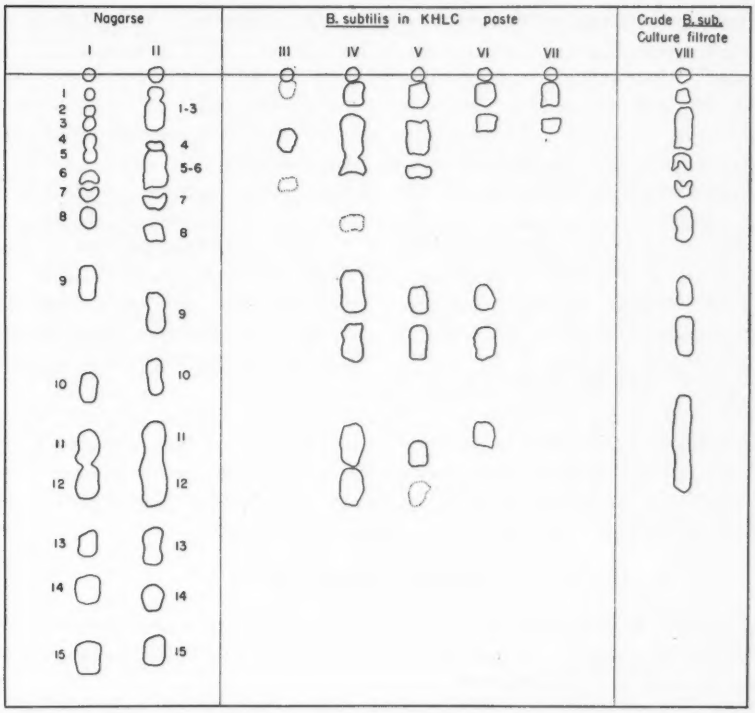

FIG. 3. One-dimensional R_f plots of chromatograms of proteolysis products of heat-denatured lysozyme. Rows I and II: Nagarse acting on lysozyme solution. Rows III through VII: *Bacillus subtilis* N′ grown on KHLC paste. Growth time: row III, time zero; row IV, 18 hours; row V, 2 days; row VI, 3 days; row VII, 6 days. Row VIII: lysozyme digest by crude filtrate of the *B. subtilis* N′ culture medium. The pattern in row I was obtained by chromatography for 18 hours in order to obtain good separation of the spots with small R_f values; chromatography time for rows II through VIII was 6 hours.

7, 13, 14, and 15 do not appear on the chromatograms obtained from the filtrates of KHLC-bacteria paste (row IV). When a crude enzyme preparation was used to digest lysozyme, the pattern of row VIII was obtained. Spots 13, 14, and 15 are again missing, although spot 7 appears. The apparent reduction in the number of spots with time (rows III–VII) is due to decreasing concentration of proteolytic products causing decrease of color density in any spot, rather than to a change in type of proteolytic activity, and the absence of fractions 13, 14, and 15 from proteolysis products by the cel -free filtrates indicates that these fractions were not selectively assimilated by the organisms prior to the 18-hour sampling. To check whether the missing proteolysis products may be adsorbed to the kaolinite paste, a solution of proteolysis products from the lysozyme digestion by Nagarse was chromatographed after 1-hour contact with kaolinite–Celite suspension. A pattern similar to row II was obtained, that is, no digestion products were adsorbed on kaolinite.

The factors causing the differences in the proteolytic behavior of the organism from that of the pure crystalline enzyme derived from it are not known. Paper electrophoresis of commercial Nagarse at pH 5.1 in phosphate buffer showed two major components and seven other trace components. Matsubara *et al.* (17) mention that the commercial Nagarse preparation contains some autodigestion products, and that a second peak appears in boundary electrophoresis at pH 6.0. Conceivably Nagarse contains some intracellular enzyme component as a contaminant or else it contains an additional adaptive enzyme resulting from the commercial growth medium. According to the source, Nagarse was obtained from *Bacillus subtilis* N′ cultures growing on mixed protein media. In our experiments we used lysozyme as the only protein source. All inocula, however, were obtained by growing *B. subtilis* N′ on nutrient broth or nutrient agar, thus avoiding any genetic selection towards lysozyme.

The study of microorganisms in this soil-like paste system permits detailed examination of the interactions between organisms, enzymes, substrates, and soil particles. Such an experimental approach might be developed into a soil-model for study of microbiological activities comparable with those in soil but on a limited and hence more convenient scale.

Acknowledgments

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THE EFFECT OF PENICILLIN ON THE STRUCTURE OF STAPHYLOCOCCAL CELL WALLS[1]

R. G. E. MURRAY, W. H. FRANCOMBE, AND B. H. MAYALL

Abstract

Cultures of sensitive strains of *Staphylococcus aureus* were fixed with osmium tetroxide after 1–5 hours' exposure to various doses of penicillin and were embedded in methacrylate for sectioning and electron microscopy. They were compared with untreated, control cultures. The contrast of the cell wall material was increased by cutting the sections onto a solution of lanthanum nitrate.

The cells increased in size and the surrounding cell wall was thinner than normal. The main lesions appeared in the developing cell wall septa, which showed a loss in density and gross irregularity of shape. Some questionable inclusions were seen in the cytoplasm. Lysis was prevented in a medium containing 0.3 *M* sucrose and the stable spheroplasts retained a recognizable cell wall after 24 hours' exposure to penicillin. However, the septa could not be demonstrated in the cells treated in sucrose medium.

Two resistant strains were exposed to penicillin. In one, the cells showed no morphological effects; in the other, there was temporary damage to the cell septa with complete recovery.

The observations support the hypothesis that penicillin interferes with the synthesis of a cell wall component and indicate that the main point of cell wall synthesis is at the site of septum formation.

Introduction

There is suggestive evidence that penicillin exerts its peculiarly selective toxic action on the synthesis of a structural component unique to the bacterial cell wall (19).

Penicillin is bound to a lipid-containing component of the cell membrane fraction (4, 5) and it is likely that this site is the cytoplasmic or plasma membrane (6). The killing of growing cells certainly involves the osmotic barrier, shown by the prevention of lysis and the production of stable spherical forms in a sucrose medium of suitable hypertonicity, but it was realized that this involved not merely the osmotic barrier but the strength and integrity of the cell wall (10, 11, 12). Lederberg (11) suggested that "As non-growing cells are not killed by penicillin, new cell wall formation, rather than the existing wall, is the probable target". The "protoplasts" induced by penicillin retain full potential because they are capable of returning to normal growth and form when the penicillin is removed. Therefore swelling, filamentous forms, large body formation, penicillin-insensitive L-forms of certain bacteria, and lysis as morphological expressions of the effects of penicillin can be explained as interference with cell wall synthesis and the consequent inability of the cell wall to act as an "osmotic corset" in a hypotonic environment.

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Contribution from the Department of Bacteriology and Immunology, Faculty of Medicine, University of Western Ontario, London, Ontario. This work was reported to the Laboratory Section of the Caandian Public Health Association, December 1957 (16).

The biochemical evidence stems particularly from the identification by Park and his co-workers (17, 18, 19) of certain normal compounds accumulating early in the action of penicillin on sensitive staphylococci. These compounds are uridine-5′-phosphate nucleotides of an amino sugar, linked to a peptide of DL-alanine, D-glutamic acid, and L-lysine (3:1:1).

The amino sugar proved to be that unique component of bacterial cell walls that has been named muramic acid (21), which is also associated with a peptide having practically identical ratios of the same amino acids. It has been suggested that this forms a structural unit within the cell wall complex (23). This "basal structure" is present, in smaller amounts, in the cell walls of Gram-negative organisms such as *Escherichia coli* (22).

The implications are that a synthetic mechanism at or close to the cell surface is damaged with a consequent defect in the construction of the cell wall fabric. The following experiments using electron microscopy were undertaken on *Staphylococcus aureus* to see what lesions occurred in or near the cell wall during penicillin poisoning.

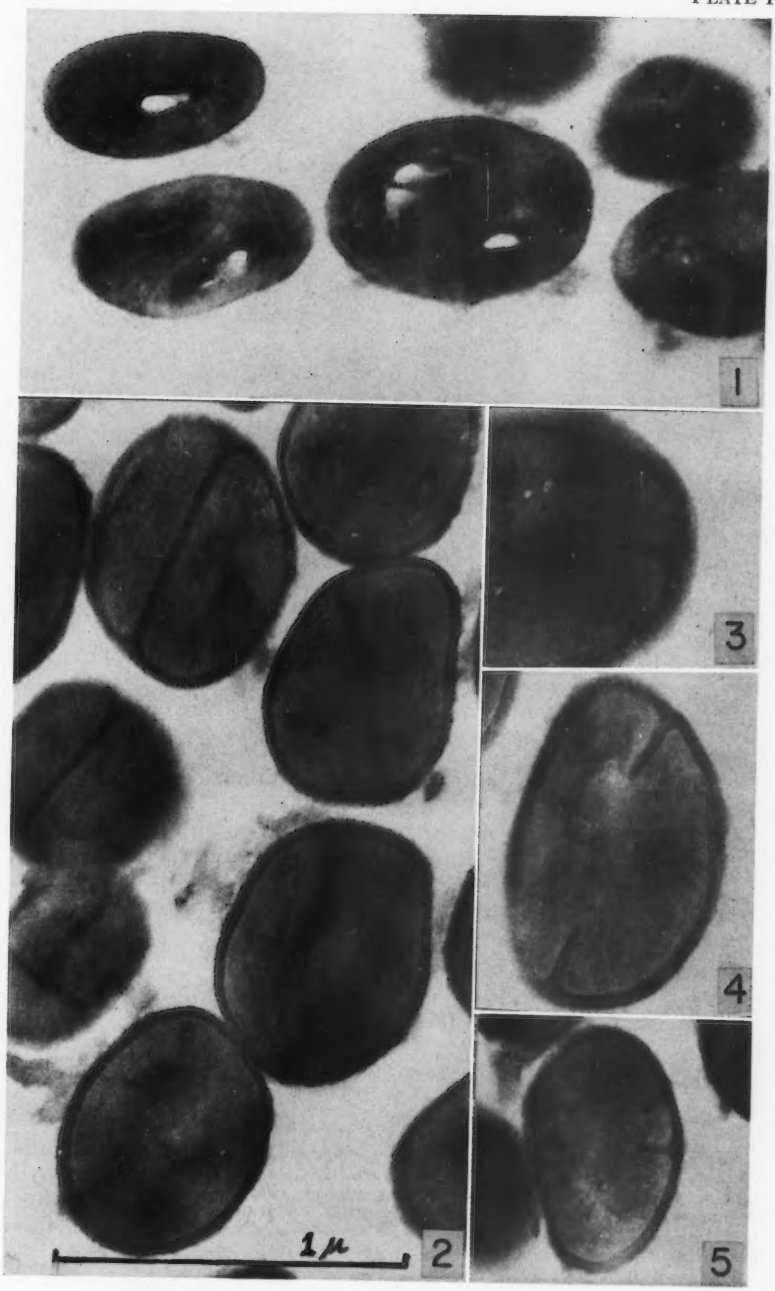
Materials and Methods

The strains of *Staphylococcus aureus* that were used in this study were isolated from specimens received in the clinical laboratory of this Department. The penicillin sensitivity of these strains was tested (using crystalline penicillin G) by the tube dilution method in penicillin assay broth (Difco) over the range of 1 to 500 μg/ml. Suitable strains were selected and thereafter designated by their clinical number. Strains 4154 and 4988 were sensitive to less than 1 μg and strain 4149 was resistant to greater than 250 μg penicillin per milliliter. Strain 4154M was selected from a series of penicillin gradient plates and was considered to be a resistant mutant of strain 4154, being resistant to greater than 250 μg/ml. These stock cultures were maintained on blood agar slopes and were transferred at weekly intervals.

In most of the experiments the cells were grown in 20-ml volumes of the laboratory nutrient broth, a papain digest broth (1), in a water bath at 37° C and with aeration by bubbling from an air pump. An overnight culture was used for inoculum and the subculture was allowed to grow for approximately 3 hours before harvesting or the addition of penicillin. In some later experiments L B broth (tryptone 1%, yeast extract 0.5%, NaCl 1%, glucose 0.1%) was used with or without the addition of 0.3 *M* sucrose.

Cells were harvested in most of the experiments by rapid centrifugation and resuspension of the pellet in 1% osmium tetroxide in distilled water for 1 hour fixation. In other experiments, particularly those with sucrose in the medium, the osmium tetroxide was added to the medium to give 0.1%. Surprisingly, there was little trouble with lysis in the fixatives and so we did not undertake factorial experiments on the constitution of fixatives.

FIGS. 1–5. Sections of normal *S. aureus* (strain 4154) grown for 5 hours. Fig. 1 is unstained; the remainder were treated with lanthanum nitrate. Figs. 3–5 show the initials of septa in profile.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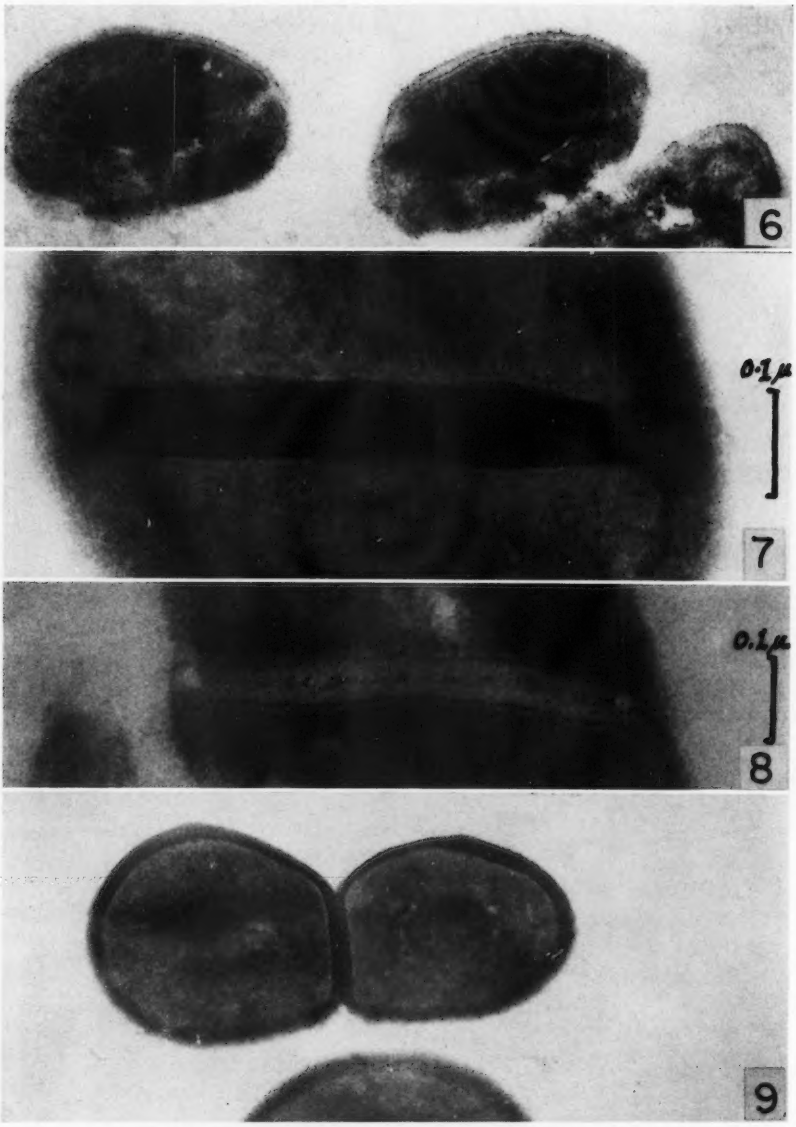

FIG. 6. Strain 4154M grown for 4 hours in tryptose broth, fixed for 1 hour with a mixture containing 1% osmium tetroxide and 0.6% potassium permanganate. Differentiation of parts of the cell membrane complex is evident.

FIGS. 7–9. Sections to show the form of the complete septum and the separation of new cells. FIG. 7. Same as Fig. 2 but higher magnification. FIG. 8. Unstained section showing formation of cleavage line and differentiation of basal layer. FIG. 9. Same as Fig. 2, showing the separation of sister cells.

The cells were dehydrated by successive spinning and resuspension in two changes of 70%, 95%, and absolute ethyl alcohol. After two washes in a mixture of equal parts of absolute alcohol and *n*-butyl methacrylate, one wash in pure *n*-butyl methacrylate, the cells were held in a mixture of *n*-butyl methacrylate containing 10% methyl methacrylate for several hours. The final embedding mixture was the same as the latter but 10% of a previously polymerized mixture was dissolved in it. The cells were suspended in this syrup and placed in gelatin capsules, which were suspended 12 inches from an ultraviolet lamp (Hanovia 500 Watt U.V.S.) for 6 hours. This was usually adequate to produce good polymerization but if further hardening was necessary the capsules were incubated at 60° C for a day or two.

Sections were cut on a Porter-Blum microtome and were collected on 10% acetone or on 1% lanthanum nitrate in distilled water. The latter was the most useful because of the gain in cell wall contrast (8, 13); the sections were best collected onto grids from below and the grids washed after collection with a few drops of distilled water. The grids were covered with a thin formvar film for support. The sections were viewed and micrographs taken with a Philips electron microscope 100-A, modified by a substitution of high-resolution objective pole pieces.

Observations

Normal Cells

In all experiments control batches of cells, which had not been exposed to penicillin but were treated identically in other ways, were embedded and sectioned. The normal cells of all strains were remarkably similar and a single description will suffice. Good cell preservation was obtained and there was little problem with severe embedding damage.

Lanthanum "staining" provided excellent contrast for the cell wall (Figs. 1–5), which stood out as a remarkably regular and fairly thick (approximately 250 Å) membrane consisting of two distinct strata. The outer region was a wide and almost homogenous layer. Directly apposed to it was a thinner (approximately 50 Å) and more dense inner zone, and immediately inside this there was a light, low-scattering zone of about the same thickness (Figs. 6–8).

It was suspected that the inner dense zone was a component of the cell wall because it seemed to be adherent to the major portion of the cell wall when separation from the cytoplasm occurred. However, incontrovertible plasmolysis was not attained and so the identity of the two thin layers is uncertain. It was felt that the inner low-scattering zone might be the site of the cytoplasmic membrane.[2]

These multiple layers at the cell surface were the most distinctly shown in preparations fixed in a mixture containing 1% osmium tetroxide and 0.6%

[2]Bacterial lipids in recognizable regions of the cell, as identified by suitable staining methods using Sudan black, do not show much density to electrons even after fixation with osmium tetroxide. This is the behavior one might expect of saturated lipids. The lipid-rich cytoplasmic membrane seems to behave in like manner (15). But this is likely to be a *relative* rather than absolute density to electrons, conditioned by the extreme density of the surrounding structures.

potassium permanganate. This treatment reduced the contrast of the massive outer zone and brought the two thin inner zones into sharp relief (Fig. 6).

The various phases of cell septum formation and cell division could be readily identified. The initials of septum formation were remarkably regular and pointed intrusions into the cytoplasm (Figs. 3–5). This intrusive septum seemed to involve all the layers of cell wall. Following the closing of the septal diaphragm, the septum thickened to approximately double the cell wall thickness when it developed in its center a finely vacuolated zone (compare Figs. 2, 7, and 8), forming a line of cleavage for the separation of the two sister cells (Fig. 9). The thickness of the peripheral cell wall seemed to remain remarkably constant throughout the active growth of the organisms and this despite the considerable increase in cell size.

No special study was made of cytoplasmic structure. The impression was gained that fine resolution of the cytoplasm was obscured by some diffuse scattering material. This was perhaps supported by the fine granular appearance of the cytoplasm in those cells fixed with the osmium–permanganate mixture mentioned above, which seems to be destructive to some cell components. In part the fluffy appearance and the seeming density of the cytoplasm were produced by the treatment of the sections with lanthanum nitrate. However, this was not the whole problem because untreated sections rarely showed any reasonable resolution of cytoplasmic architecture.

Sensitive Strains Exposed to Penicillin

The sensitive strain 4154 was exposed for 1, 3, $5\frac{1}{2}$, and 24 hours to penicillin at a concentration of 20 μg/ml.

Two changes were immediately apparent in the cells that had been exposed for 1 hour: the cell size increased, and the cell wall seemed to be thinner and showed irregularities that were not apparent in the control cells (Fig. 10). In particular, the developing cell septa did not show such a regularly pointed intrusion; they had become more spadelike and diffuse at the edges. Even some of the apparently complete transverse septa had irregular margins and were paler than in the normal cells.

At 3 hours the lesions were much more evident (Fig. 11). The cells were much larger than normal and this coincided reasonably well with light microscope observations. Cell walls in the peripheral part of the cell were markedly thin and the partially developed septa were blunted and misshapen; in some places they even seemed to be reduplicated. A number of dark cytoplasmic bodies were visible in the lanthanum-stained preparations and these were not present in the control. These cytoplasmic and cell wall changes were hardly discernible in the sections that had not been treated with lanthanum nitrate; in fact, it was difficult to tell whether septa were present or not. From the marked lanthanum uptake of the cell wall components and of the abnormal material in the cells, it seemed probable that the two were related. The peripheral cell wall was still visible in the untreated sections; it was the septum and the area immediately adjacent to the septum that was of such low

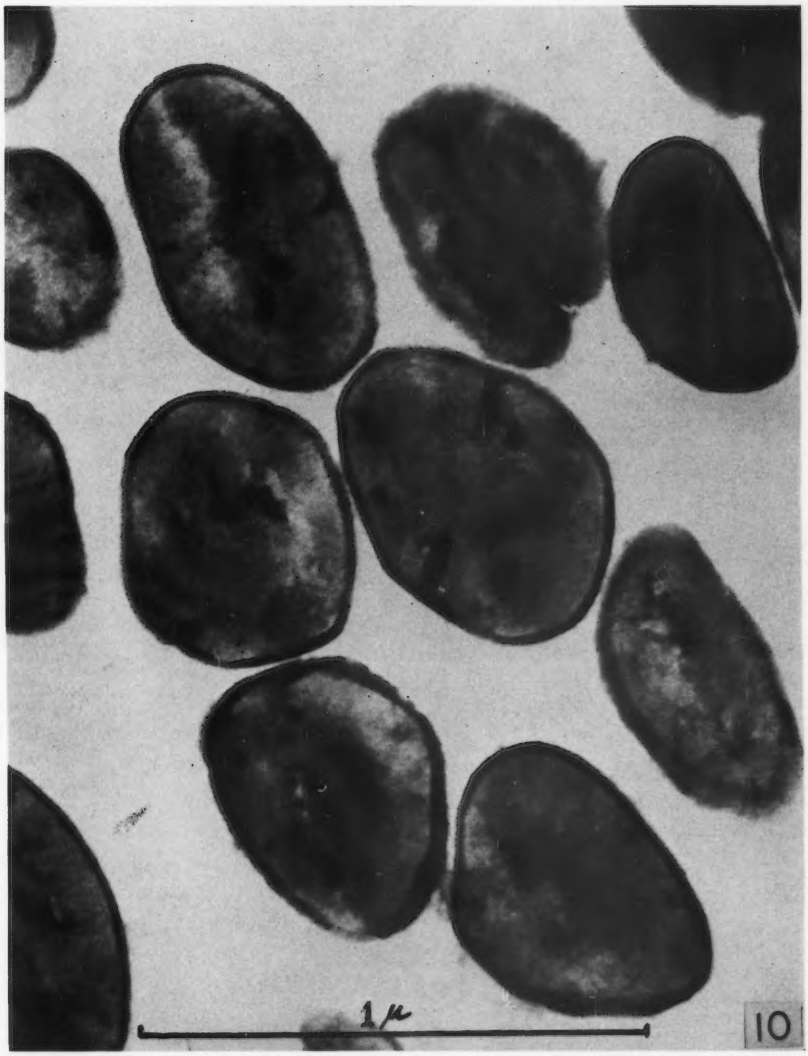

FIG. 10. Sections of *S. aureus* (strain 4154) exposed to 20 μg/ml penicillin for 1 hour. Section treated with lanthanum nitrate.

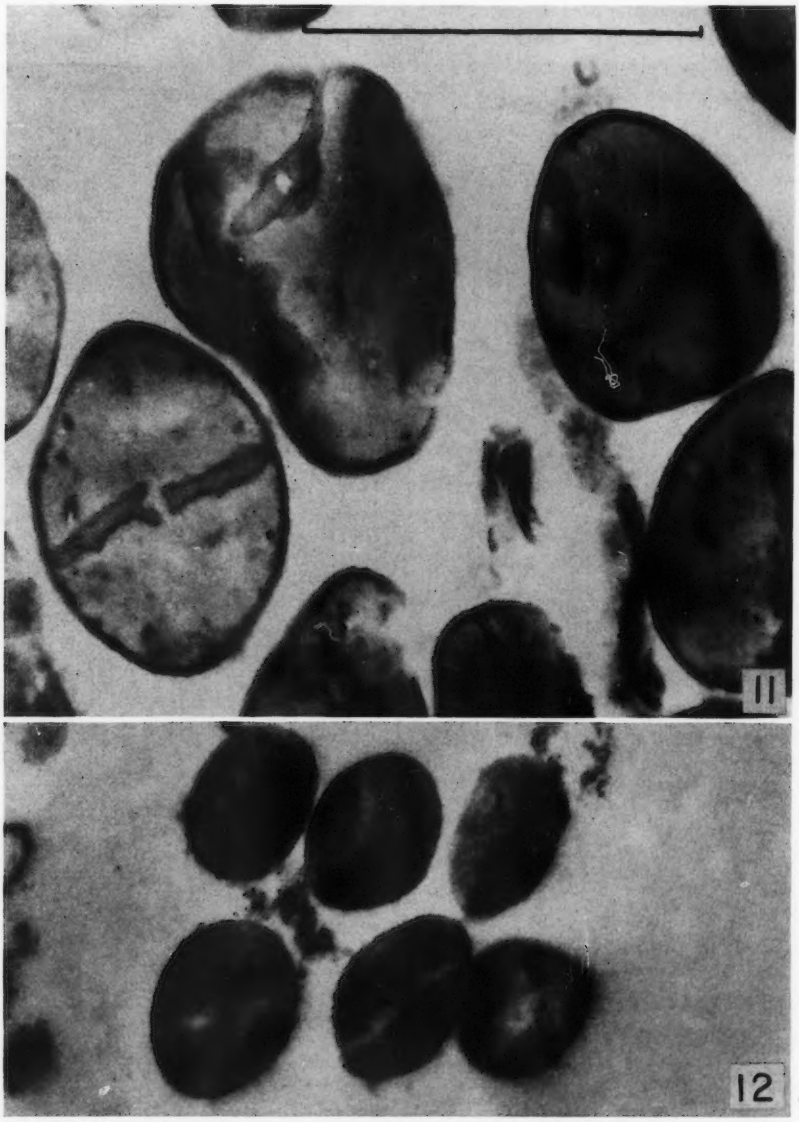

FIG. 11. From same experiment as Fig. 10 but exposed to penicillin for 3 hours. Section treated with lanthanum nitrate.

FIG. 12. From same embedding as Fig. 11 but section not treated with lanthanum salt, and lower magnification.

FIG. 13. Same as Fig. 11.

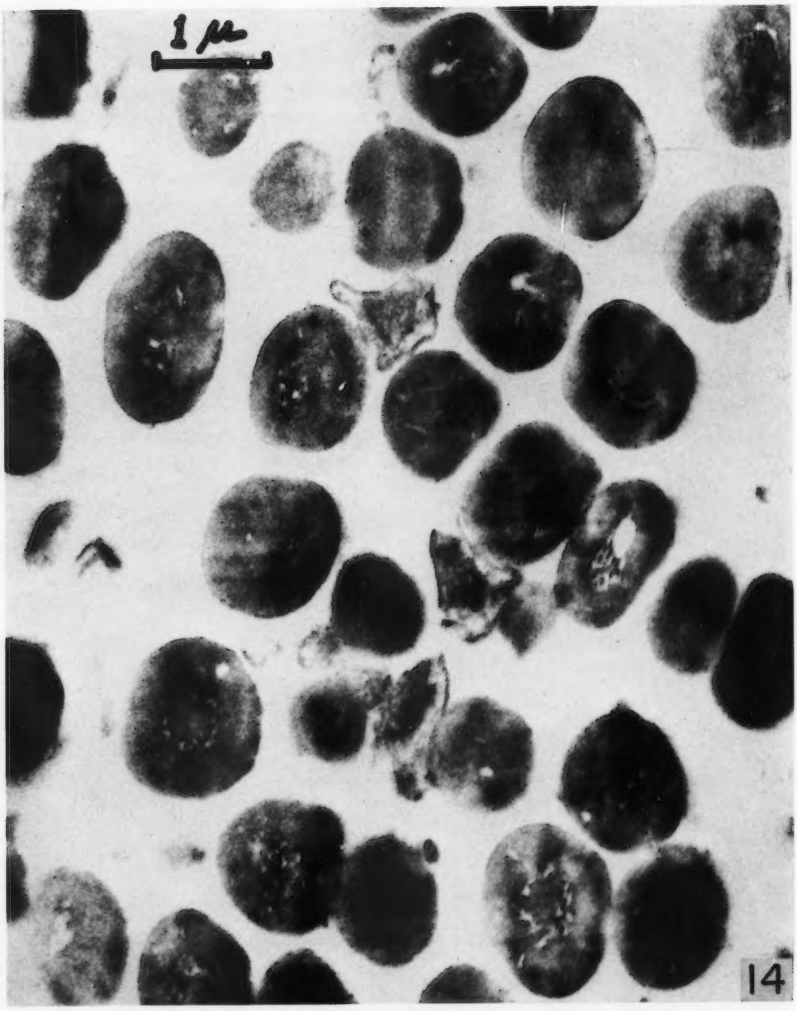

FIG. 14. Print from a low-power survey micrograph of *S. aureus* (strain 4988) exposed to 20 μg/ml penicillin for 6 hours in an environment containing 0.3 *M* sucrose. The section was treated with lanthanum nitrate and deformed (or normal) septa were not seen. The cell diameters approximate to twice that of normal cells; the cell wall approximates ¼–½ usual thickness.

contrast (Fig. 12). This indicated that the major synthesis of the cell wall takes place in the septal portion. The peripheral cell wall must be presumed to stretch with the swelling of the cell.

At the 3-hour interval there were indications of cellular lysis because a number of empty and fragmentary cell walls appeared in the preparations. This was very marked in the 5½-hour preparations and here it was noted that some of the surviving cells seemed to be of almost normal configuration. The few cells that were left in this rather high concentration of penicillin for 24 hours were markedly abnormal.

Sensitive strain 4988 was exposed to smaller amounts of penicillin: 0.1, 1.0, and 20 μg/ml of penicillin. The larger amount produced substantially the same effects as before in the same time interval. The smaller amounts, particularly 0.1 μg/ml, produced much less clearly defined structural effects. It was evident after 3 hours that there was a similar form of distortion of the initials of septa and completed septa to that recorded above and there were similar inclusions in the cytoplasm.

Resistant Strains Exposed to Penicillin

The resistant strain 4149 was exposed to 20 μg/ml of penicillin for 1, 3, and 5½ hours.

After 1 hour virtually all the cells were abnormal and the appearance of the cell walls closely resembled that already shown on sensitive strains after an equal exposure. Some of the cells were larger and more irregular than those in the controls, but in the over-all appearance it was seen that the total proportion of damaged cells was lower than when sensitive strains were similarly treated. The peripheral cell wall thickness was variable but thinner (150–200 Å) than in the controls. In the 3-hour preparation abnormal cells were still present although many approached normal appearance. By 5½ hours recovery seemed to be complete and no real points of distinction could be found between these cells and their controls. There were few cell wall lesions, lysis was not obvious, and the cell size approximated to normal. There seemed to be a slight lag in the growth of the cultures but after the 3-hour interval growth continued unimpeded.

The resistant strain 4154M was exposed to 100, 500, and 1000 μg/ml penicillin for 5 hours and in no case did the cells show the gross changes that were apparent in the sensitive strain or the changes seen in the resistant strain 4149. It thus seemed that selected penicillin-resistant strains reacted somewhat differently to penicillin in their environment; perhaps this was due to variation in the capacity for adaptive production of penicillinase.

Effect of Penicillin in a Hypertonic Medium

The production of osmotically sensitive, swollen forms by treatment with penicillin in a hypertonic medium allowed determination of the longer term effects of penicillin (7). There was no doubt that 0.2–0.3 *M* sucrose prevented lysis and led to the production of enlarged spherical forms that survived and remained intact for upwards of 24 hours. These forms were shown to be sensitive to osmotic lysis by dilution of the medium.

The spherical forms or "spheroplasts" (3) certainly retained a recognizable cell wall over the entire period of observation although this was undoubtedly thinner than normal. The untreated controls, in the presence of sucrose in the medium, showed a perfectly normal appearance consistent with that seen in the previous experiments.

The cells were markedly enlarged after 1 hour exposure to 20 μg/ml penicillin and the peripheral cell wall was certainly one-half to two-thirds the normal thickness. However, a most puzzling feature was that neither normal nor abnormal septum formation was visible in the sections. This occurred in several preparations (Fig. 14). The lanthanum uptake in the remaining cell wall was not as marked as before. Although there were some very pallid areas traversing the cell, which might have represented the positions of septa, they could not be defined even with "staining". The cytoplasm seemed to show a considerable lanthanum uptake. It was realized that a tryptone medium was employed, thus the experiments differed from those of previous sections in more respects than the addition of sucrose. Extraction and even metabolic modifications were considered but further experiments were not performed. However, these observations did indicate, again, that a result of penicillin poisoning was a dislocation of cell wall synthesis at the "growing point", the septum.

Discussion

There is no doubt that penicillin can induce changes in the cell wall structure of staphylococci. The thinning of the peripheral cell wall seems to be the expression of stretching due to the increase, up to twofold, in the diameter of the cells without concomitant cell wall growth. There seems to be a disturbance of the regular laying down of new cell wall material in the developing septum and this disturbance involves not only the laying down of material of lower density (or lower scattering power for electrons) but also a distortion of the geometry of the structure. There is some indication that inclusion pockets containing material of similar density and reaction to lanthanum nitrate can occur in the cytoplasm, although one must realize that these could be cuts through projection from a distorted septum or projections from the cytoplasmic surface at some other site (Figs. 11, 13).

It seems reasonable to assume that the components forming the cell wall are incorporated and polymerized *in situ*. They are probably manufactured at some site in or close to the cytoplasmic membrane. It is most unfortunate that our present technical methods in electron microscopy do not allow us to make a defined study of the cytoplasmic membrane and the part that it may play in this process (15). However, the extremely regular inner table of the cell wall and the surface of the cytoplasm, which is applied to it in the normal cell, compared with the fine irregularities of the same region in cells treated with penicillin gives us a very slight indication of disturbance in the relations of the two.

It was surprising to find that one of the two resistant strains studied reacted to penicillin in a similar way to the sensitive cells. There was the marked difference that the cells did not go on to lysis but recovered rapidly and resumed growth. The fact that another strain did not show this sequence of changes indicates that some strains may have more capable means of dealing with penicillin in their environment. Resistant strains of *Staphylococcus* vary considerably in their ability to produce penicillinase when they have not been grown in a penicillin-containing medium (9). It might be that we were observing the lesions occurring up to the point that the adaptive production of penicillinase was sufficient to deal with the penicillin in the environment.

The observation that cells, inhibited by penicillin but maintained in a reasonably stable state by a sufficient concentration of sucrose in the environment, retained a definite cell wall structure for upwards of 24 hours is indicative that penicillin does not cause dissolution of the existing cell wall or interfere with the total cell wall structure. This has been discussed elsewhere in greater detail (19, 22). The penicillin effect seems to involve a basal component of cell wall structure that is involved with the strength and the shape of this specialized container. The observation reaffirms the decision already taken (3) to consider that penicillin-induced "protoplasts" are not equivalent to those in which the cell wall structure can be removed entirely by treatment with lysozyme in a suitable environment. At least the term "spheroplast" does not imply any confusing connotations.

It has been suspected that the synthesis of bacterial cell walls does not occur diffusely but rather is concentrated at some "growing point". There have been ingenious approaches to decision on this matter using flagella as cell wall markers, but the results have not been concordant (2, 20). The observations on the induction of large bodies of *Escherichia coli* and *Proteus vulgaris* with penicillin show that the point of cell wall weakness occurs at the site of cell division and only in growing cells (11, 12). The autolysis of staphylococci leads to the release of two hemispherical portions of wall, this separation being accomplished by dissolution along a weakened line that might correspond to the equator of the succeeding division (14). These sorts of observation, together with our own, indicate that cell wall synthesis is concentrated in that portion of cell wall that is to accomplish the separation of sister cells.

Acknowledgments

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FACTORS AFFECTING THE VIABILITY OF AIR-BORNE BACTERIA

I. BACTERIA AEROSOLIZED FROM DISTILLED WATER[1]

S. J. WEBB

Abstract

The death of bacterial cells aerosolized from distilled water suspensions has been studied over a 5-hour period. It was found that death occurred in two stages, a rapid initial kill taking place within the first second and a subsequent slower death. First-order kinetics only applied to this secondary death at low relative humidity ranges, and this is taken to indicate that at least two reactions are responsible for viable decay. The death rates of the air-borne cells have been correlated with a mathematical function of both temperature and humidity. Activation energies associated with aerosol death have been found to increase from 4000 cal/mole to 12,000 cal/mole as the cells age in air. It is suggested that the death of the cell results from the movement of water molecules in and out of the cell, in an equilibrium system, resulting in a collapse of the natural structure of cellular protein.

Introduction

Studies on the transmission of infection through the air at a distance greater than that envisaged under the term "droplet infection" have been conducted over the past two decades and have become the subject of two reviews (2, 27). The effect of relative humidity (R.H.) and temperature on the air-borne cells has formed the greater part of these studies, but there still seem to be differences of opinion, between workers, on these effects. Some researchers have stated that the death of the air-borne cell is greatest at high R.H. levels (4, 18, 33), some reported contrary findings (32), whereas others claim intermediate R.H. levels to be the most lethal (5, 7, 28). More recently, attempts have been made to distinguish between real and apparent death due respectively to loss of viability of the air-borne cells and particulate settling (6). The results of these experiments have indicated that loss of viability was greatest at low R.H. levels. However, the electronic method of calculating total cell numbers, used in these studies, was only applicable to bacterial aerosols generated from distilled water. In 1958 Harper *et al.* (10) reported the use of radioactive cells in aerobiological studies and during the past three years the author has employed a radioactive tracer technique to determine total cell numbers in aerosols. It is the purpose of this paper to describe the technique and its subsequent use in the study of bacterial aerosol behavior.

Materials and Methods

I. Organisms

Serratia marcescens (Fort Detrick strain 100)
Escherichia coli (type 1, University of Alberta)
Staphylococcus albus (University of Alberta)
Bacillus subtilis (Fort Detrick strain)

[1]Manuscript received June 22, 1959.
Contribution from the Bacteriology Section, Defence Research Board of Canada, Suffield Experimental Station, Ralston, Alberta (DRB Project No. D52-18-50-04).

II. Culture of Organisms

All organisms were cultured in 2% Bacto-Tryptose plus 1% dextrose on a rotary shaker at 30° C.

III. Preparation of Radioactive Tracer Cells

The organism to be studied was grown in the above medium but containing also 20 mc of P^{32}/100 ml of medium. After 48 hours of growth 0.5 ml of a saturated solution of iodine in 5% KI was added per 100 ml of medium, and the culture allowed to stand for 15 minutes. The cells were then harvested by centrifugation at 8000 r.p.m. and washed with 0.85% NaCl (saline) until the supernatant radioactive (R.A.) count per 0.1 ml was down to 5 counts per minute (c.p.m.). In this way dead radioactive cells were obtained that were stable in that the P^{32} did not leach from the cells on storage or during aerosolization and collection. The cells were finally resuspended in saline and stored at 4° C.

IV. Preparation of Spray Suspensions from which the Aerosols were Generated

(*a*) *For Rapid Decay Studies 0 to 10 Seconds of Air Suspension*

Using a Coleman No. 9 nephelocolorimeter, a standard curve was obtained by plotting cell numbers per milliliter of distilled H_2O against optical density. From this graph a suspension of the organisms to be studied was prepared containing 1×10^7 viable cells/ml. One milliliter of this suspension was placed in 18 ml of distilled H_2O, contained in a Collison spray bottle (12). To this suspension 1 ml of the tracer cell suspension was added. A spray suspension containing 5×10^5 viable cells/ml was found to be the best concentration as it allowed viable counting to be carried out directly from the impinger samples without diluting.

(*b*) *For Slow Decay Studies* (*up to 5 Hours of Air Suspension*)

Suspensions were prepared by spinning out, at 8000 r.p.m., the cells from 20 ml of a 24-hour culture and 20 ml of the tracer cells suspension. The pellet of tracer cells was resuspended in 20 ml of distilled water and the whole of this suspension was added to the pellet of viable cells. In this way a suspension was obtained containing approximately 1×10^{10} cells/ml each of tracer and viable cells.

V. Aerosol Generation and Storage

(*a*) *Aerosol Generation*

The aerosol was produced in all cases by operating a Collison spray (12) at 26 p.s.i. This spray produces droplets of 5 microns (μ) mean diameter.

(*b*) *Storage up to 10 Seconds*

The Collison spray outlet was connected to a modified Henderson apparatus consisting of a copper tube 2 in. in diameter and 5 ft 6 in. in length through which air was drawn at a rate of 19 liters/minute by means of a vacuum pump. One-inch diameter ports served as sampling points at 6-in. intervals along the tube, thus the air storage time between each port was 1 second. The 2-in. tube was surrounded by an outer tube of 4-in. diameter through which water of varying temperature could be passed. The circulating air, carrying the air-

borne cells, was passed through the 2-in. tube by the pump, then through two bacterial filters, three control valves situated on a control panel, and a control valve, which allowed some air to escape to the exterior via a fume cupboard, and then through a humidifying–drying apparatus, and finally was returned to the aerosol tube 4 in. from the Collison spray outlet. The flow rate was adjusted by operating the control valves and observing mercury manometers on the control panel. The whole apparatus was, therefore, a partially closed system.

(*c*) *Storage up to 5 Hours*

The prepared suspension was atomized by a Collison spray and the aerosol carried into an 1100-liter steel drum, similar to that already described (9), rotating at 4 r.p.m. The humidity inside the drum was previously set, by passing in dry or wet air, and was measured by means of a Serdex humidity gauge. Samples were taken from a 1-in. tube leading from the interior of the drum, immediately after a 5-minute atomizing period and at subsequent hourly intervals. A second drum was housed in a refrigerated room where temperature could be reduced from 25° C to −40° C. Facilities were not available to study temperatures above 25° C with the rotating drum.

VI. Sampling

One-minute samples were collected from the aerosol tube at 9 liters/minute in all-glass Porton liquid impingers containing 6 ml of 0.85% NaCl (saline). Samples from the drums were taken for 1 minute at 12.5 liters/minute in Shipe glass impingers containing 10 ml of saline. The design of the aerosol tube was such that the Porton impinger was easier to use, whilst the drum design favored the Shipe. In preliminary experiments no difference in viable count of a liter of an aerosol could be detected when both types of samplers were used to assess the same aerosol.

VII. Total and Viable Cell Counting

The initial suspensions were serially diluted in saline and 0.1 ml of each dilution was plated on Bacto-Tryptose agar to obtain the viable count. Similar aliquots (0.1 ml) of each dilution were placed in a 1-in. diameter aluminum planchet each containing a disk of Whatman No. 1 filter paper. The planchet samples were placed on the automatic sample changer of a Tracerlab end window R.A. counting apparatus.[2] The Autoscaler was set to count 1000 counts and the time for this number of counts was automatically recorded, to two decimal places, by a timer. The ratio of R.A. counts/0.1 ml of dilution to viable count/0.1 ml of the same dilution was determined and used to calculate the total cell numbers in impinger samples from equation 1.

$$\text{Total cells} = \frac{\text{No. of viable cells/0.1 ml of spray suspension}}{\text{R.A. counts/0.1 ml of spray suspension}} \times \text{R.A. count/0.1 ml of collecting fluid.} \quad (1)$$

All viable counts were obtained by the drop plate technique on Bacto-Tryptose agar.

[2]Tracerlab model No. SC6C.

Results

When bacterial cells were aerosolized from distilled water suspensions a large number of cells were found to die within a second, and the subsequent death during the next 9 seconds was relatively slow. The extent of this death is plotted in Fig. 1A, where it can be seen that the sensitivity to aerosolization differed markedly from organism to organism. Only 20% of *E. coli* cells survived this period, whereas 75% of *S. albus* cells remained viable. These results differed only in rate from those of other workers (6), the extent of death during 10 seconds of air storage being approximately the same. In order to compare these data with Ferry's work it was necessary to apply first-order kinetics to the death curve by using equation 2.

$$K = \frac{1}{t} \log_e \frac{\text{total cells/liter of air } (T)}{\text{viable cells/liter of air } (V)} . \qquad (2)$$

$\text{Log}_e (T/V)$ was plotted, therefore, against t (in seconds) and results are shown in Fig. 1B. It was evident that first-order kinetics did not apply to the whole 10-second period, but that a straight line was a good fit to the points after the 1-second period and it seemed reasonable to characterize the two periods by decay constant K_0 (0 to 1 second) and K_s (1 to 10 seconds) to facilitate further comparisons. The same phenomenon was encountered when the death

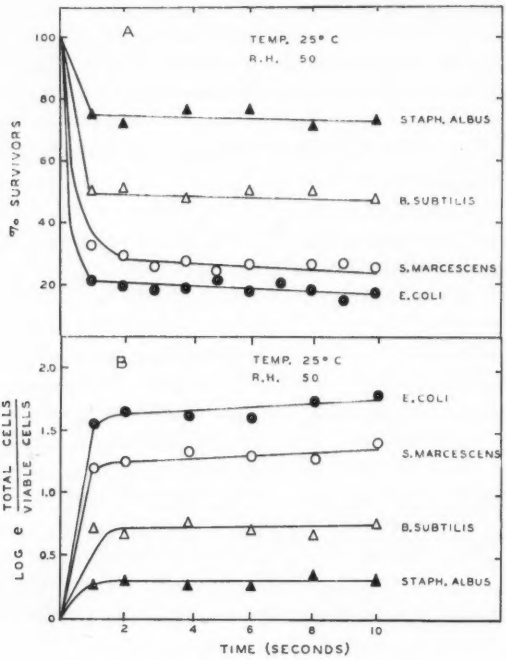

FIG. 1. Rapid death from 0 to 10 seconds.

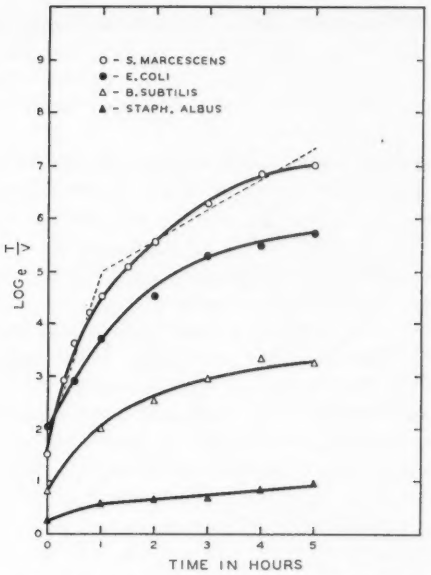

FIG. 2. Death from 0 to 5 hours. R.H. 50%; temp. 25° C.

of cells at 50% R.H. was studied over a 5-hour period. The plot $\log_e (T/V)$ against time (in minutes) was a curve, and again it was necessary to choose arbitrary times to define K values. Since Ferry had studied periods of about one hour, this period was defined as K_1 and from 1 hour to 5 hours as K_4. In Fig. 2, it can be seen, from the dotted lines, that if only 1 hour of air suspension was studied, a straight line was a reasonable fit to the points. The results also demonstrated that the same trend existed between genera as in the earlier stages. *E. coli* and *S. marcescens* were far more susceptible to air storage than either of the other two organisms.

TABLE I

Comparison of death rates of various organisms at 50% R.H.

	M. candidus	*S. albus*	*E. coli*		*S. marcescens*		*B. subtilis*
			Ferry	Webb	Ferry	Webb	
K_{10}	0.051*	0.031	0.20*	0.17	0.15* 0.061*	0.13	—
K_1	0.003*	0.006	0.021*	0.032	0.030	0.036	0.020
K_4	—	0.0003	—	0.010	—	0.011	0.0005

*From Ferry *et al.* (6). K_1 corresponds to Ferry's $K_2^1 \times 10^{-2}$, K_{10} = Ferry $K_1^1 \times 10^{-2}$ and is the value obtained if the first 10 seconds is treated as a first-order reaction (Fig. 1B). K_{10} is expressed in seconds. K_1 and K_4 are expressed in minutes.

By treating the whole of the first 10-second air storage period ($K_0 + K_s$) as an exponential function, defined by rate K_{10}, and by treating the first hour likewise (K_1), it was possible to compare results with Ferry *et al.* (6). The comparisons are tabulated in Table I where results are shown to be in good agreement.

The fact that the decay rates, calculated by different techniques, compared so favorably was taken to indicate that the radioactive tracer cell technique, for calculating total cell numbers, was as efficient as the electronic light scatter method. This made possible later studies on the effect of various chemical additives to the spray suspensions since the fall-out of drops, due to physical phenomena, could now be accounted for in calculations.

Having established that technique was satisfactory, studies of the effect of temperature and humidity were continued. When cells were aerosolized at various R.H. levels and at constant temperature of 25° C it was found that the K value for each period of air suspension behaved slightly differently according to the R.H. of the air. For the K_0 period, the decay rate decreased as R.H. increased up to 75% (Fig. 3A) but above this level of R.H., a sudden increase was observed in K_0 values. This simulated the findings of Ferry *et al.* (6). In the K_s period the decay rate decreased with increasing R.H. and cells exhibited maximal stability at 70% R.H. and above (Fig. 3B). The relationship between the decay rates over the first hour and those for the subsequent 4 hours was extremely interesting. It was found that K_1 remained

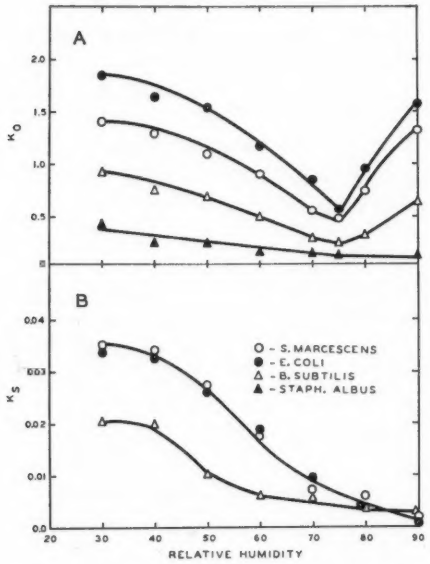

FIG. 3. The variation of K_0 and K_s with changing R.H. (T, 25° C).

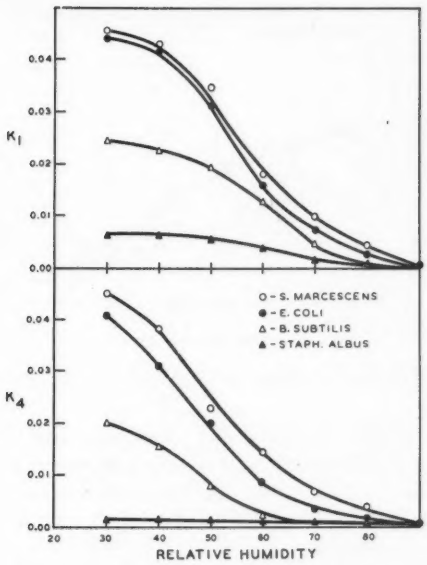

FIG. 4. The variation of K_1 and K_4 with changing R.H. (T, 25° C).

fairly constant from 20 to 50% R.H. and then rapidly fell at 60% R.H. and over. K_4 values, however, decreased from 40% R.H. and above. Thus at 30% R.H. and below, K_1 and K_4 had approximately the same value, indicating that first-order kinetics did apply to death below 30% R.H. (Figs. 4A and 4B). This suggested to the author that the mechanism responsible for death consisted of two or more reactions taking place simultaneously or at different periods but that one was more affected by R.H. than the other.

The sudden rise in K_0 values at 75% R.H. was difficult to understand. However, it has been reported that freeze-dried cells of *S. marcescens* were killed when allowed to take up finite amounts of water, by rehydration in air of various relative humidity levels (20), and this death termed "sorbed water killing". It seemed possible, therefore, that the sudden rise in K_0 was due to the same phenomenon since the 5μ diameter drops, put out by the Collison spray, would evaporate within microseconds (31). By using wet and dry bulb thermometers placed in the top of the spray apparatus the R.H. of the air inside the spray bottle was found to be 73%, when dry air (R.H. 5) was used to generate the aerosol. It was subsequently observed that when the air, returning to the aerosol tube, via a tube 4 in. from the spray outlet, was above 75% R.H., a rise in K_0 occurred. When the humidity of the air generating the aerosol was raised, the point at which K_0 increased moved towards a higher R.H. level and with mixing air of 90% R.H. no rise in K_0 was observed (Fig. 5). This "rewetting" phenomenon was also observed in the drums.

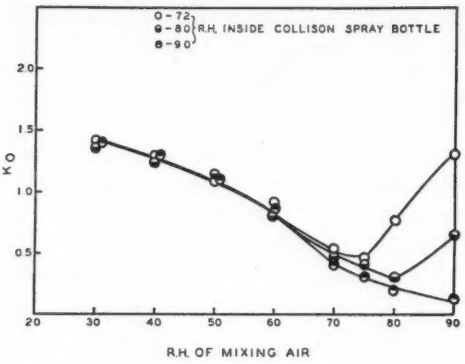

FIG. 5. The effect of mixing air humidity on K_0 for *S. marcescens*.

If the humidity in the drum, containing an aging aerosol, was raised by 5%, an immediate drop in the number of viable cells was encountered. It was important, therefore, in subsequent experiments to ensure that the humidity of the air, in which the cells were to be suspended, was always below that of the air in the spray apparatus.

To study temperature effects cells were first aerosolized at 50% R.H. and at varying temperatures, and with the exception of K_0 all rates were found to increase as the temperature increased. K_0, however, became smaller with temperature rise. In Fig. 6 the effect of temperature on K_0 is shown in a typical Arrhenius plot, $\log_e K_0$ being plotted against the reciprocal of the absolute temperature and Fig. 7 demonstrates by the same method the temperature effect on K_s. These data were plotted in this fashion, since K_s appeared to follow first-order kinetics and it seemed reasonable to assume K_0 did likewise. From this type of graph it was possible to calculate the activation energy (E) from the slope of the line (E/R) associated with the death mechanism and also the activation entropy (ΔS), from equation 3 (30), assuming E to be equal to ΔH (26).

$$K = \frac{k_b T}{h} e^{\Delta S/R} - e^{\Delta H/RT} \qquad (3)$$

K = death rate of cells
k_b = Blotzmann's constant
h = Planck's constant
T = absolute temperature
ΔS = activation entropy
ΔH = heat of activation
R = gas constant

This approach has been taken by many enzymologists and virologists. It was necessary, however, to bear in mind that the sum total of all reaction was probably being measured and secondly that it was being determined by

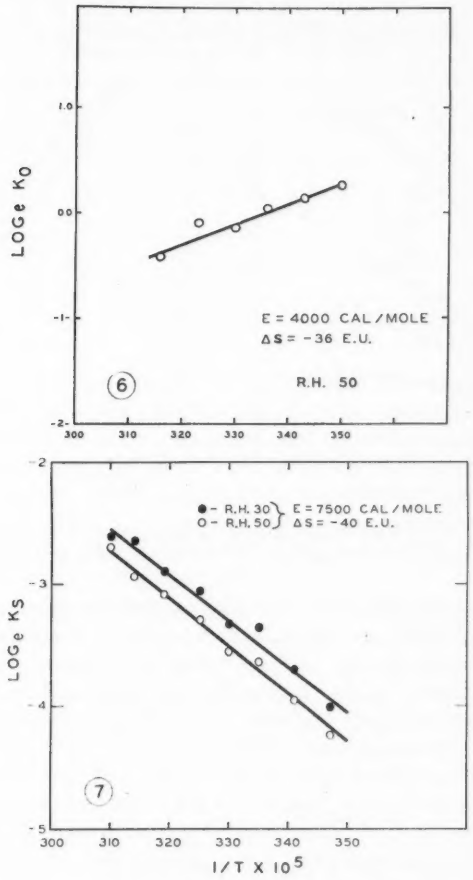

FIG. 6. Variation of K_0 with temperature for *S. marcescens*.
FIG. 7. Variation of K_s with temperature for *S. marcescens*.

using the death of the cell as an end point. Thus it was unlikely that an increase in temperature would slow a reaction and produce the lower K_0; what was more likely was that a faster reaction rate governing K_0 could give the cell a better chance of survival. Since it had been found that at 30% R.H. first-order kinetics applied to the cellular death during the first 5 hours of air suspension in the drums, it was decided to study the effect of temperature, on the death of cells during long air suspension times, at this R.H. level.

Unfortunately with the available drum apparatus it was not possible to study temperatures above 25° C, so that only a study of lower temperatures could be made. It was found that during this 5-hour period the rate of death

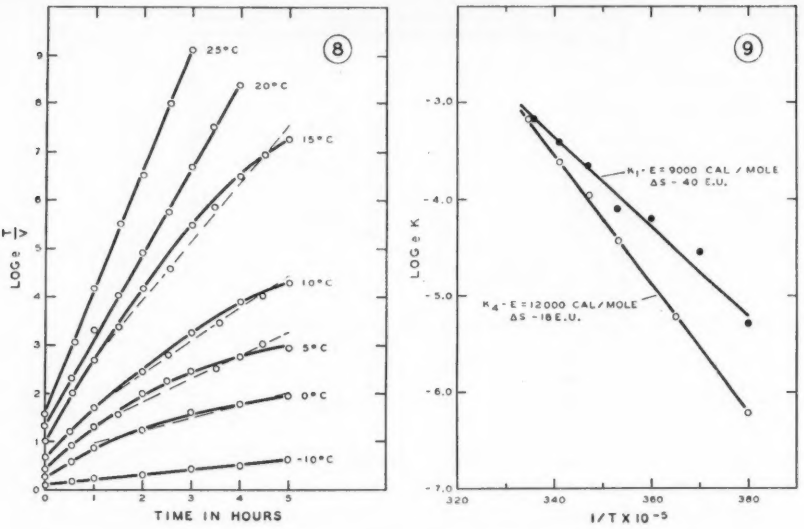

FIG. 8. The effect of temperature on the death of *S. marcescens.*
FIG. 9. Effect of temperature on K_1 and K_4 for *S. marcescens.*

slowed as temperature decreased but that K_4 was the more affected. Thus, as was found with R.H. effects, K_1 and K_4 seemed to separate below 20° C and above 0° C and the plot $\log_e (T/V)$ between this temperature range appeared to be a gradual curve (Fig. 8). Below 0° C very little cellular death occurred and therefore, any difference between K_1 and K_4 periods was obscured and a straight line relationship seemed to hold over the full 5-hour period. As first-order kinetics only applied to the death of air-borne cells under some environmental conditions, it was felt that two or more reactions were involved in the death mechanism but that they were of a first-order type. This type of difficulty has been encountered by many protein chemists and biologists and has resulted in some workers splitting up reactions into phases. Since at temperatures of 15° C, 10° C, and 5° C, the difference between the curved line and straight line plots (demonstrated by the dotted lines in Fig. 8), for the K_4 period, was within experimental error, it seemed reasonable to compare the E values of this period with those of the K_0, K_s, and K_1 periods. The results are shown in Figs. 6, 7, and 9, from which E values were obtained of 4000 cal (K_0), 7500 cal (K_s), 9000 cal (K_1), and 12,000 cal (K_4). The activation entropy (ΔS) of approximately -40 E.U. did not change during the first three periods, but rose to -18 E.U. for the K_4 period. The activation energy appeared to be unaffected by R.H. as is shown in Fig. 7, where two plots are shown of the effect of temperature on K_s at 30 and 50% R.H. It was evident then that the longer the cells remained air-borne the greater was the amount of energy required to kill them. The same studies on *E. coli* and *B. subtilis* gave similar values of activation energies tabulated in Table II.

TABLE II

The activation energy associated with cellular death during four time periods of air suspension

	E values (cal/mole)			
	K_0	K_s	K_1	K_4
S. marcescens	4,000	7,500	9,000	12,000
E. coli	3,500	7,000	8,500	10,000
B. subtilis	3,800	6,600	7,000	7,500
S. albus	—	—	—	—

Staphylococcus albus showed no effect of temperature below 35° C for the K_0 and K_s periods, or below 25° C during the subsequent two periods, whereas the death of *B. subtilis*, although affected by temperature, showed more resistance to heat than either of the two Gram-negative species. The smaller *E* values for *B. subtilis* death results from the method of their calculation. By using the death rate in an Arrhenius plot and evaluating *E* from the slope, it follows that the less affected the cells are by temperature, the smaller will be the slopes and the calculated *E* values.

From the above results, it was evident to the author that, since the cells were being aerosolized from distilled water, the concept of death by osmotic pressure changes in the drying drop could not be used to explain the data. The rapidity of death of some genera at low R.H. and the stability of *Staphylococcus albus* cells seemed to suggest that death resulted from some intrinsic property of the cell structure and that this was in some way connected with the relationship between the cell and water vapor of the air.

In order to ascertain whether the death of the cells was a function of humidity and temperature combined, the formula for the evaporation of water drops in air (equation 4), devised by Frössling (8), was utilized as shown below:

$$-\frac{dm}{dt} = \frac{4\pi MD}{RT}(P_0-P)rF \qquad (4)$$

$$-\frac{dm}{dt} = A\left(\frac{P_0-P}{T}\right) \text{ for drops of equal size}$$

since $$\text{R.H.} = \frac{100P}{P_0}$$

$$-\frac{dm}{dt} = A(100-\text{R.H.})\frac{P_0}{T}$$

where D = diffusion constant of vapor in air
F = function of Reynolds Number for drop diffusion and is constant for drops of equal size

M = molecular weight of droplet material
P_0 = saturated vapor pressure at T
P = partial vapor pressure at T
R = gas constant
r = radius of drop
T = temperature ° K
m = droplet mass
t = time in seconds
R.H. = relative humidity

It has been shown that small drops evaporate almost instantaneously (31) so that it was felt that by applying this formula to the death of bacteria, aerosolized from water, function f $((100-\text{R.H.})(P_0/T))$ could represent the rate of change of water between the aerosol drop and the surrounding atmosphere. A good fit to a sigmoid curve was obtained when the K_s death rates of the cells, air-stored under various conditions of R.H. and temperature, were plotted against function f (Fig. 10). Due to the limitations of the temperature range of the drums, values of f above 6 could not be obtained during the K_1 and K_4 periods of air storage. However, over this limited range of temperature the plots of K_1 and K_4 against f appeared as a straight line (Fig. 11, A and B). It was noted that, in all plots, as the values of f became smaller the spread of the individual points from the line became larger. This was thought to be due to the fact that at low temperatures R.H. had very little effect on the death rate and thus the relationship between K and f values would not hold.

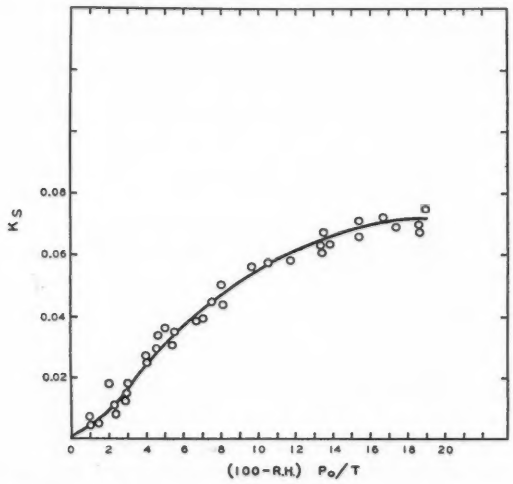

FIG. 10. Variation of K_s with function f for *S. marcescens*.

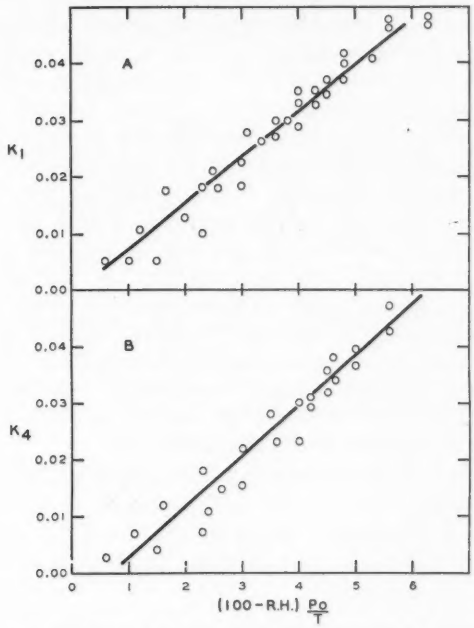

FIG. 11. The variation of K_1 and K_4 with function f for *S. marcescens*.

It was evident from the plots of K values versus f and the fact that temperature rise increased the value of P_0/T, that at higher temperatures in order to maintain K at a low level an increase in R.H. was necessary. Thus the death rate of the cells during air storage appeared to be determined by a combined effect of both temperature and humidity.

At this stage of the investigations no intermediate conditions of R.H. and temperature had been found to be optimal for cellular aerosol death, and it was felt that these optima, observed by other workers, could have been due to the effects of the constituents of the medium from which the cells had been aerosolized.

Discussion

The stability of air-borne cells has been found, by many workers, to differ from genus to genus and the studies reported in this work support these earlier findings. There does seem, however, to be a common factor affecting the viability of the air-borne cell and this appears to be the relationship between the cellular proteins and water.

The method of representation of the data by means of four decay rates is open to criticism. However, since a large number of publications have been presented in this manner, it was necessary, for the sake of comparison, to analyze the presented results in a like fashion. Also, as was subsequently found, it enabled any differences in cellular death, with respect to time, to be detected.

The extent of the rapid initial killing of cells aerosolized from water during the first second of atomization agrees favorably with the results of Ferry *et al.* (6) and also with observations by Scott (29) on cells of *Salmonella newport* dried in sealed ampules. The difference in rate of this kill can possibly be explained by the smaller size of the aerosol droplets produced by the Collison spray as compared with those produced by the apparatus of Ferry *et al.* It is evident that the rate of removal of water during this period of death can affect the extent of death and it would seem that since a rise in temperature decreased the death, the more rapidly the water is removed the better is the chance of cellular survival. This contrasts with the findings of Scott (29), who found, for capsule-dried cells *in vacuo*, that the slower the water was removed the greater was the survival. However, marked differences were noted by Scott between *vacuo*-dried cells and those dried in air. As Scott was studying death over weeks of storage, it is possible that the period of rapid kill he reported is in fact the K_s and K_1 periods of death reported in this paper. Certainly time scales were widely different.

The effect of R.H. on the cells atomized from distilled water is similar to the results of Ferry *et al.* (6) in that no intermediate R.H. level favored death. It is noticeable that all periods of death followed a similar pattern and this is taken to indicate that death can be a direct result of the protein–water relationships of the air-borne cell.

It is evident, from the effect of temperature, that a difference exists between the kill during the first second of aerosolization and that during subsequent time periods. This difference is probably due to the fact that the first second is an equilibration period, whereas later death is concerned with changes in equilibrium. This hypothesis is supported by the fact that rapid increases in R.H. produce an immediate kill of cells in an aged aerosol, and since this R.H. effect could be detected during the K_0 period of air suspension, it must be assumed that the equilibration process is extremely rapid and takes place within microseconds. This would be expected since the rate of evaporation of water drops, determined by Langstroth *et al.* (14, 15) and Wells (31), is equally rapid.

In summing up his results, Scott (29) states that "Apparently the removal of the most firmly held water molecules results in some loss of viability, especially in air". Although in the present studies no difference could be found between cellular death in air or inert gases, it is possible that, in the studies of Scott (29), on long-term survival, some basal metabolism continued which eventually resulted in the death of the cell. This, however, could not occur to any detectable extent in 5 hours of air storage and presumably plays no part in the death of the air-borne cell. It was noticed, in these studies, that a few cells, representing only 0.01% of the initial cell concentration in the aerosol, remained viable for 48 hours or longer. However, when these cells were collected and recultured the same pattern of aerosol behavior was produced. It would seem, therefore, that in any one population of cells a small number are able to survive the adverse conditions of air storage probably

indefinitely. It is interesting to note here that if the survival of the cell was a matter of chance, its chances of survival would be approximately 1 in 10^4 cells, and this is approximately equal to the number of degrees of freedom (1×10^4) of an average protein molecule (21).

That the death of the cell is connected with a dynamic system rather than a static system is suggested by the apparent relationship between the cellular death rate and a mathematical combination of water vapor pressure and temperature represented by function $f((100 - \text{R.H.})(P_0/T))$. The relationship shown indicates that at high R.H. and low temperatures little death would occur, and in fact this was found to be so.

The interpretation of this relationship, however, can be made in two ways:

(*a*) that at low temperatures and high R.H. the equilibrium water content of the cells would be high and

(*b*) the rate of exchange between the cellular protein's 'bonded water' and the water vapor of the air would be extremely slow.

Taking the two variables independently, there can be only one effect of R.H. and that is to determine the number of protein–water molecules that are lost or exchanged, the rate of loss or exchange would not alter, unless the bound water of the cell was involved and the strength of the protein–water bonds varied from bond to bond. Temperature, on the other hand, would affect the rate of loss or exchange. Studies on the vapor tension of bacterial cells with respect to temperature would help to elucidate this problem. Nevertheless it is difficult to explain why an air-borne cell in equilibrium with the atmospheric water vapor of its environment should die, and fairly rapidly, if a dynamic system of water molecule exchanges is not visualized. It is postulated, therefore, that in the aerosol a dynamic system exists as below:

$$\text{protein} + H_2O \text{ vapor} \underset{KP + H_2O}{\overset{K_p\ H_2O}{\rightleftharpoons}} \text{protein, } H_2O, + \text{energy.}$$

In such a system, if a set number of water molecules per protein molecule are necessary to maintain structure, it follows that equilibria, with exchange rates that allow periodically too few protein–H_2O bonds, will produce death. Thus at high values of f, the chances are increased of a periodic state occurring where there are too few bonded water molecules. It is also possible to visualize that, if an equilibrium is disturbed, by the addition of small amounts of water (e.g. by increasing R.H.), a state of too few protein–water bonds could be produced if the heat energy resulting from the bonding of vapor to some protein sites is greater than the energy level of other protein–water bonds.

The fact that first-order kinetics does not apply to the death of the air-borne cells at 50% R.H. but does at 30% R.H. indicates that death is governed by at least two reactions. If the death of the cell is due to the loss of bonded water, it follows that the difference between the apparent order of reaction at the two R.H. levels is due to the dependence of the exchange, on the relative strengths of the protein–water bonds.

In any one protein molecule there are many sites for water bonding and there must be also a large variation in the strengths of the protein–water bonds due to the differences in affinities for water between the protein groups. It would be expected, therefore, that, in proteins in their normal aqueous environment, a gradation of water–protein hydrogen bonds from weak to strong would exist. It is possible to visualize, therefore, that in an air-borne cell the rate of exchange of bonded water with atmospheric water vapor is determined by the strength of the protein–water bonds and the production of death may require the rupture of one hundred weak bonds but only two or three of the stronger bonds.

Following the removal of water some irreversible change may well take place in the protein structure. If this change is produced by a tightening of the protein helix through irreversible protein–protein bonds, it follows that the chances for these protein–protein bonds to occur are greatly increased by an increased exchange of water molecules. Since the speed of weaker bond exchanges would tend to suppress the extent of stronger bond exchanges, by keeping the vapor concentration in the air relatively constant, it is possible that at 25° C and 50% R.H. death due to strong bond exchanges could be slight. At high R.H. however, there must come a time when all possible irreversible changes in protein structure, due to the weaker exchanges, have taken place and the subsequent rate will be a measure of the extent of strong exchanges. At lower R.H. (30%) more of the strongly held water would become involved, and although the rate of water exchange is unaffected, the chances of lethal strong bond exchange deaths occurring are greatly increased.

There are innumerable permutations of such a system, and because the death of the cell is used as an end point, only when the numerous rates of exchange are such that each produces death at the same rate would the plot $\log_e (T/V)$ against time appear as a straight line.

It is possible that the above explanation could account for the lack of agreement, between workers, on the order of the reaction determining the death of cells and perhaps the denaturation of some proteins. Partial orders and first- to fifth-order reactions have been postulated, and it seems reasonable that where death or biological inactivity is used as an end point in rate determinations, the same reaction going on in different parts of the protein molecule may produce widely different effects. The rupture or formation of one bond may not affect biological activity, whereas a similar make and break of a bond in another part of the molecule may cause its inactivation.

The fact that exchange rate as well as the number of water molecules is involved in death is indicated by temperature effects. There is the peculiar phenomenon of decreased death with increased temperature during the first second of aerosolization. This rapid initial kill may also be connected with the removal of the first bonded water molecules and it is possible that the faster the water molecules are removed the less chance there is of a lethal change in structure. There is, however, another explanation of this phenomenon. It has been shown that cells of *E. coli* are rapidly killed by sudden chilling

to 4° C (19), and since the evaporation of the aerosol water drops would result in a sudden lowering of temperature at their surface, it is possible that some of the initial aerosol kill is produced by a large temperature drop. Hence when the process of evaporation is carried out at higher temperatures the chilling effect is less.

Subsequent cellular death is affected by temperature in the same way as it is by R.H. When temperature is lowered the plot $\log_e (T/V)$ against time assumes the form of a curve and with further lowering of temperature it resumes its straight line characteristics. This can be explained by the assumption that some exchange rates are far more affected by temperature than others. Thus some would quicken or slow to a greater extent than others with the same change in temperature. It is possible, therefore, that death during the K_1 period of aerosolization is governed by fairly rapid exchange rates which have, at 25° C, reached such a speed that increases in rate will not increase the cellular death rate. When temperature is lowered, however, the exchange rate is still sufficient to produce rapid death, but the rates governing the K_4 period have slowed sufficiently to produce only a small death, thus the first-order plot becomes a gradual curve. At still lower temperatures all exchange rates have slowed sufficiently to produce death rates of equal magnitude in both the K_1 and K_4 periods of air storage.

A great deal of criticism has been leveled at the use of thermodynamic studies in biological investigations. Most of the critics insisting that the reaction being studies is usually not an isolated system. However, there is no doubt that studies of this kind have yielded valuable information in enzymology, virology, and biochemistry in general. It is true that the application of thermodynamic principles to biological experiments cannot be as exact as their use in pure physical chemistry, until systems are better understood. However, they can be used in biology on a comparative basis, to indicate the nature of reactions relative to one and another and to others of a more defined nature.

The studies reported in this paper were viewed critically in order to determine whether a thermodynamic analysis of the results would yield any useful information. The kinetics of denaturations and inactivations have been reviewed (22) and it was found that deviations from first-order kinetics occurred in about one third of the studies. Critical analysis showed also that there were few kinetic studies with highly purified proteins and it is well known that impurities may affect even the order of the reaction. In more recent studies with purer proteins some denaturations have appeared to follow first-order kinetics (11, 13, 17), some second order (1), and others partial orders (16), in some cases the apparent order depended on the method of estimating the velocity (26). This is a clear indication that there are numerous stages in protein denaturation. Where biological activity or function is used as the basis of determining rates, the possible reversal of some inactivations must be taken into account and it should also be realized that loss of activity may consist of a series of concomitant or consecutive changes, any one of which may pro-

duce inactivity and thus determine velocity. It is evident, then, that any experimental quantity measured is a property of one or more reactions and constitutes the sum total of all effects.

Reviewing the first-order plots of $\log_e (T/V)$ against time, it was considered that during the K_3 and K_1 periods of aerosolization the death of the air-borne cell followed first-order kinetics, when measured by the techniques available. If the K_4 period of death was isolated from the K_1 period a straight line was also a good fit to the experimental points, and at certain humidity and temperature levels the plots were all definitely first order. In view of the very minor deviation from first-order plot at 30% R.H. and 15° C and by the obvious lack of true first-order kinetics in numerous publications, it was considered that useful information could be obtained by treating each of the decay periods as first order and analyzing them thermodynamically. In this way a comparison between the four death periods would reveal any differences, and also allow results to be compared with those of other workers. To calculate entropy changes the death rates used were those where death during all of the five-hour period of air suspension fitted a first-order plot (i.e. at 30% R.H. and 25° C or 30% R.H. and 20° C).

It is evident that the activation energy (E) for death increases with age of the air-borne cell, and for a given rise in temperature death during the K_4 period is the most affected. These results suggest that, as the cell ages in air, more energy is required to produce its death, but that, if this energy is available, the rate of death during the later periods of aerosolization increases more rapidly than that during the earlier stages. This seems to fit in with the hypothesis that death is due to the rates of exchange between water vapor and protein-bonded water molecules. If these rates are governed by the relative strengths of the protein–water bonds, it seems reasonable that E values would increase with aerosol age. Also if the rupture of the stronger bonds was more lethal than the breakage of weaker bonds, it follows that an increment of say a factor of two in the number of bonds broken could result in a small increase in the K_1 death rates and a large increase in the K_4 death rates. The value of E during this period of death also agrees with a figure of 7000 cal/mole found, for sorbed water killing of *S. marcescens* (20) and thus it would seem that the E value for death is the same for both water adsorption and desorption.

The negative entropy of aerosol death indicates that the process of death involves a tightening of the protein molecules. However, since the entropy calculated represents the sum total of events some loosening of structure may also occur. The loss of water should in itself result in a positive entropy and it is of interest to compare the activation entropies calculated with those of other workers (24, 25).

From the above comparison there is one striking fact and that is the greatly reduced values of E and negative values of $\triangle S$ for the inactivation of micro-organisms in the dry state. As Pollard (23) points out, the entropy does not seem to be related to the size of the organism, which means that inactivation

TABLE III

The activation energy and entropy
for the inactivation of microorganisms

Organism	Condition	$E(\Delta H)$, cal/mole	ΔS	Reference
T-1 phage	Dry	27,500	0	Pollard and Reaume (1951)
	Wet	95,000	207	
T-2 phage	Dry	18,000	−12	Pollard and Reaume (1951)
	Wet	71,000	139	
T-3 phage	Dry	19,100	−9	Pollard and Reaume (1951)
	Wet	105,000	246	
T-7 phage	Dry	12,700	−29	
TMV	Dry	27,000	0	Pollard and Dimond (1953)
SBMV	Dry	17,000	−16	Pollard and Dimond (1953)
S. marcescens	Aerosol (K_1)	9,000	−40	Webb
	Aerosol (K_4)	12,000	−18	
E. coli	Aerosol (K_1)	8,500	−38	Webb
	Aerosol (K_4)	10,000	−19	
S. lactis phage	Wet 30–45° C	11,000	—	Cherry and Watson (1949)
	55–65° C	76,000	—	

is taking place in relatively small regions and is indicative of structure and function in the cells. It is evident, too, that the values of E and ΔS for aerosol death during the K_4 period are of the same order as those reported for the inactivation of dried viruses. From the data of Cherry and Watson (3) it appears that the measured E values can also depend on the range of temperature studied. This is another indication that inactivation is governed by more than one reaction for at high temperature ranges (55–65° C) the activation energy is much higher than at 30 to 45° C. Again loss of viability was the criterion used to measure the velocity of the reaction, and if one reaction was unable to take place at 30 to 45° C or take place too slowly to cause death, the death rate would be slow. However, if the other reaction was more affected by temperature, in that small changes in its rate produced large changes in death rate, a temperature increase in the 55 to 65° C range would produce far more deaths and hence death rate. The pattern of virus inactivation, therefore, follows that of the death of air-borne cells and it is possible that the two phenomena are similar.

The magnitude of E and ΔS seems to indicate that the changes taking place in the cell are of a subtle nature. The denaturation of most proteins by heat involves values of E of over 160,000 cal/mole and entropies of around 300 entropy units (E.U.). Even though the measured values for bacterial death must be considered the result of the sum total of reactions, this total is small since it has been estimated that an increase of 100 E.U. corresponds to a

change of only 0.5% in the vibrational frequency for a molecule with 10,000 internal degrees of freedom (21). However, most of the heat inactivations have been carried out at relatively high temperatures (60 to 80° C) and it has been shown (3) that E and ΔS can be a function of the temperature range studied. Pollard (23) also states that the wet heat inactivation of *Tobacco mosaic virus* yields two sets of figures. Below 85° C E = 40,000 and ΔS 18 and above 85° C E = 195,000 and ΔS 410 E.U. This indicates that two very different processes are involved in denaturation at low and high temperatures. It is possible, therefore, that the difference between the E and ΔS values of inactivations of wet and dry cells at low temperatures is due to the relative amounts of water in the cell. Thus the positive ΔS and higher E values of wet inactivations are due to the necessity of removing considerably more water molecules from sites on the protein. Naturally, if there were several water layers covering one site, a possible attack on the vital site would be more difficult than if no such protective layer existed. This in itself speaks strongly for the stabilizing action of water in protein structure.

The small values of E would also suggest that bonds of a low energy level were involved in the death mechanism. Such bonds would probably be ionic, hydrogen, or phosphate bonds and since all are present in protein structure all three may take part in the death of the cell and it is felt that the study of the effects of various chemical additives on the death of the air-borne cells might indicate the nature of the structure concerned.

Acknowledgment

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CORRECTIONS

Volume 4, page 612. In Solution B, the quantity of $CuCl_2.2H_2O$ should read 0.269 mg.

Volume 5, pages 493–503. The spelling of the species *Humicola alopallonella* should be changed to read *Humicola halopallonella*.

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